THE ROLE OF SUBCELLULAR COMPARTMENTS AND STRUCTURES IN EUKARYOTIC GENE EXPRESSION

By

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KEY TO ABBREVIATIONS

BSA	Bovine Serum Albumin
cpm	Counts per minute
dCTP	Deoxycytidine-5'-triphosphate
ddCTP	Dideoxycytidine-5'-triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Hepes	N-2 hydroxyethyl piperazine-N'-2 ethanesulfonic acid
HU	Hydroxyurea
µCi	Microcurie
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
OD	Optical density
PVS	Polyvinyl sulfate
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecylsulfate
SSC	Standard saline citrate buffer
Tris	(hydroxymethyl)aminomethane

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Histone mRNAs naturally reside on nonmembrane-bound polysomes that are associated with the cytoskeleton. To study whether the subcellular location plays a role in the coupling of histone mRNA stability to DNA synthesis, a signal peptide-histone fusion gene was constructed that encodes a histone mRNA that is directed to membrane-bound polysomes. The signal peptide-histone fusion mRNA when localized in the foreign subcellular compartment is no longer destabilized when DNA synthesis is inhibited. A single nucleotide substitution in the signal peptide-histone fusion gene by site-directed mutagenesis resulted in the localization of the histone fusion mRNA on nonmembrane-bound polysomes and restored the coupling of mRNA stability with

DNA synthesis. These results indicate that the subcellular location of histone mRNA plays an important role in the posttranscriptional regulation of histone gene expression.

We have determined that the association of histone mRNA with the cytoskeleton is dependent on the integrity of actin filaments. Disruption of the microfilaments with cytochálasin D efficiently released histone mRNA from the cytoskeleton into the soluble phase. In contrast, membrane-bound polysomal mRNAs remained attached to the cytoskeleton during cytochalasin D treatment. Subsequent studies have demonstrated that membrane-bound polysomal mRNAs are attached to the cytoskeleton at two different sites: 1) a cytochalasin D sensitive site and 2) a puromycin sensitive site.

Disruption of the cytoskeleton with cytochalasin D resulted in a rapid and marked stimulation in transcription of the c-fos proto-oncogene and an increase in c-fos mRNA cellular levels. Transcription of several other genes was unaffected by the cytochalasin D treatment. These results suggest that the nucleus can respond to the structural organization of the cytoskeleton and selectively adjust the regulation of gene expression accordingly.

CHAPTER 1

INTRODUCTION

General Background

Histones are small basic proteins that are involved in the packaging of eukaryotic DNA into chromatin. It is estimated that more than 2 meters of DNA are contained within the limited area of the nucleus. The condensation of the DNA to such a great extent is due primarily to its interactions with the histone proteins. In addition to their structural properties histones play a functional role in controlling gene transcription. The way in which the histone proteins are arranged along a region of DNA appears to affect the transcriptional properties of that DNA domain. The complex nature of the regulation of histone protein synthesis at the transcriptional, posttranscriptional, and posttranslational levels makes these genes an important model for studying eukaryotic gene expression. However, one of the most important reasons for studying the regulation of histone gene expression is that the synthesis of the majority of histone proteins (cell cycle dependent histones) occurs during DNA replication. Elucidation of the mechanisms coupling histone protein synthesis with DNA synthesis may

yield a better understanding of the processes controlling proliferation, differentiation, cell aging and neoplasia.

Histone Genes and Proteins

There are five major classes of histone proteins (H4, H2B, H2A, H3, and H1) ranging in molecular weight from 11,000 to 21,000 Daltons. They are basic proteins that have been highly conserved throughout evolution. For example, there are only two amino acids out of 102 which differ between histone H4 from pea seedlings and calf thymus, which suggests a critical role for these proteins in cell survival (DeLange et al., 1969). The histone proteins are encoded by a family of moderately repeated genes (Wilson and Melli, 1977; Kedes, 1979). In humans, the histone genes are arranged in clusters that are not organized in simple tandem repeats as found in the sea urchin and other lower eukaryotic genomes (Heintz et al., 1981; Sierra et al., 1982; Carozzi et al., 1984). The clusters are localized on chromosomes 1, 6, and 12 and are interspersed with repetitive elements (Green et al., 1984b; Collart et al., 1985). It has been speculated that these repetitive elements may play a role in recombination and gene duplication during the evolution of this multi-gene family. The general structure of the human histone genes is less complex than many other eukaryotic genes since the histone protein coding region is not interrupted by introns and the encoded mRNAs

are not polyadenylated (Adensik and Darnell, 1972). However, the genes coding for histone proteins that are synthesized in the absence of DNA synthesis (cell cycle independent histones) generally contain introns and code for mRNAs that are poly-adenylated (Engel et al., 1982; Harvey et al., 1983; Brush et al., 1985; Wells and Kedes, 1985). Subsequent discussions will generalize what is true only for the cell cycle dependent histones unless otherwise indicated.

Histone Gene Regulation: Transcriptional and Post-Transcriptional Control

The stringent requirement to package newly replicated DNA into chromatin, the invariable 1:1 molar ratio of histone protein to DNA and the inability of most eukaryotic cells to store unbound histone proteins implies a temporal and functional coupling of histone protein synthesis with DNA replication (Prescott, 1966; Allfrey et al., 1963). Support for this hypothesis first came from studies on synchronized cell cultures of Euplotes eurystomus in which the synthesis of histone protein was found to occur only during the S phase of the cell cycle, the time when DNA replication takes place (Prescott, 1966). Furthermore, the quantity of histone protein within the cell doubled during S phase. The temporal coupling of histone protein synthesis with DNA synthesis has since been confirmed and well documented by a variety of direct experimental approaches

(Robbins and Borun, 1967; Spaulding et al., 1966; Stein and Borun, 1972). In vitro translation studies using polysomal RNA isolated from cells at various stages of the cell cycle indicated that the histone mRNA was present exclusively during S phase (Spalding et al., 1966; Gallwitz and Mueller, 1969; Jacobs-Lorena et al., 1972; Gallwitz and Briendl, 1972; Perry and Kelley, 1973; Borun et al., 1975). Quantitation of histone mRNA content during the cell cycle by Northern blot analysis, using radiolabeled histone cDNA and genomic clones, demonstrated that histone mRNA steady levels closely parallel the rates of histone protein synthesis as well as the rates of DNA replication (Heintz et al., 1983; Plumb et al., 1983a; Baumbach et al., 1984). The timing of histone protein synthesis during DNA synthesis is not coincidental and represents a functional relationship between these two processes. This is supported by the following observations: 1) there is a coordinate induction of histone protein synthesis and DNA synthesis during stimulation of quiescent cells to proliferate (DeLisle et al., 1983; Green et al., 1984a); 2) inhibition of DNA synthesis with compounds such as hydroxyurea or cytosine arabinoside results in a loss of histone mRNA with a concomitant decrease in histone protein synthesis (Breindl and Gallwitz, 1973; Butler and Mueller, 1973; Borun et al., 1975; Baumbach et al., 1984) and 3) terminal differentiation is marked by a down regulation in histone protein synthesis

in conjunction with the decrease in proliferative activity (Bird et al., 1985; Challoner et al., 1989; Stein et al., 1989).

The expression of histone genes during the cell cycle is regulated at both the transcriptional and posttránscriptional levels. Analysis by hybrid selection of RNA radiolabeled in synchronized HeLa cells demonstrates a burst of 3- to 5- fold in transcription of the histone genes within the first two hours of S phase, followed by a reduction to a basal level that remains constant until the following S phase (Plumb et al., 1983a). Similar results were obtained when histone gene transcription during the cell cycle was measured by nuclear run-on analysis (Sive et al., 1984; Baumbach et al., 1987). Steady state levels of histone mRNA, however, were elevated 15-20 fold during S phase and closely paralleled the rates of DNA synthesis (Plumb et al., 1983a; Heintz et al., 1983; Baumbach et al., 1984). Maximal accumulation of histone mRNA occurred at 5 hours into S phase and returned to a basal level as DNA synthesis ended. The timing and extent of the increase in histone transcription can not account for the marked increase in histone mRNA steady state levels and must be a consequence of a posttranscriptional control mechanism. Posttranscriptional regulation of histone gene expression is further supported by DNA synthesis inhibitor studies. The estimated half life of histone mRNA in exponentially growing

HeLa cells is 45-60 minutes (Sittman et al., 1983; Plumb et al., 1983). Hydroxyurea, or any other compound or treatment that inhibits DNA synthesis studied to date, results in a rapid and selective destabilization of histone mRNA (half life of 10-15 minutes) with little or no effect on histone gene transcription (Sittman et al., 1983; Plumb et al., 1983; Baumbach et al., 1987).

A long-standing observation concerning histone mRNA turnover during inhibition of DNA synthesis is that the mechanism is completely dependent on protein synthesis (Butler and Mueller, 1973; Gallwitz, 1975; Stahl and Gallwitz, 1977; Stimac et al., 1983; Baumbach et al., 1984; Helms et al., 1984). Pretreatment of cells with inhibitors of protein synthesis such as high salt, cycloheximide or puromycin followed by the addition of a DNA synthesis inhibitor prevents the destabilization of histone mRNAs and actually results in an increase in their steady state levels due to the continued transcription of the histone genes. This observation led Butler and Mueller and others to hypothesize that the histone proteins can autoregulate their own synthesis through a feedback loop operative at the posttranscriptional level (Butler and Mueller, 1973; Stein and Stein, 1984; Wu and Bonner, 1985). The model predicts that at the natural end of S phase or when DNA synthesis is interrupted with inhibitors a transient accumulation of unbound histone protein may arise due to a loss in high-

affinity binding sites for the newly synthesized histone proteins. Unbound histone protein may then interact with histone mRNA-containing polysomes resulting in a conformational change in the mRNA that is more accessible to ribonuclease. Growing support for this model comes from a cell-free mRNA decay system in which the destabilization of histone mRNA is accelerated when incubated in the presence of histone proteins (Peltz and Ross, 1987). This result appears to be specific for histone mRNA since the rates of decay for gamma globin mRNA, c-myc mRNA, total poly(A)$^-$ RNA or total poly(A)$^+$ RNA are unaffected by the presence of histone proteins.

Gene transfer studies demonstrated that the minimal sequence necessary for coupling histone mRNA stability with DNA replication lies within the 3' terminus of the mRNA (Stauber et al., 1986; Levine et al., 1987; Pandey and Marzluff, 1987). Removal of only 60 nucleotides from the 3' end of a murine H3 histone mRNA releases the mutant mRNA from destabilization during inhibition of DNA synthesis (Levine et al., 1987). Conversely, the last 30 nucleotides of histone H3 mRNA when fused to the 3' end of human gamma globin mRNA are sufficient to confer instability to the chimeric mRNA when DNA synthesis is inhibited, whereas wild type gamma globin mRNA remains stable (Pandey and Marzluff, 1987). Contained within the minimal target sequence for destabilization is a stem-loop structure which is common to

all known cell cycle dependent histone mRNAs (Hentschel and Birnstiel, 1981). The presence of the stem-loop structure is not sufficient by itself to couple mRNA stability with DNA replication. The target sequences must be present in the proper position at the 3' terminus of the mRNA to be operative. Aberrant histone mRNA transcripts containing the target sequences at an internal position are fully stable during the inhibition of DNA synthesis (Alterman et al., 1985; Luscher et al., 1985; Pandey and Marzluff, 1987; Levine et al., 1987). Furthermore, moving the stem-loop structure 380 nucleotides downstream from the translation stop codon abolishes the coupling of histone mRNA stability with DNA replication despite the retention of the stem loop structure at the 3'terminus (Graves et al., 1987).

An additional constraint on the histone posttranscriptional regulatory mechanism is that translation must proceed to within close proximity of the natural termination codon for destabilization to occur during inhibition of DNA synthesis (Graves et al., 1987). Frame-shift mutations in histone mRNA resulting in the premature termination of translation more than 300 nucleotides from the 3' terminus stabilize the messenger RNA when DNA synthesis is interrupted (Graves et al., 1987). Other frame-shift mutants that allow translation to continue past the natural UGA stop codon into the 3' untranslated region are also stable when DNA synthesis is inhibited (Capasso et al.,

1987). Correcting for translation read through in these mutants by inserting stop codons in all three reading frames at the natural translation termination site restores the coupling of mRNA stability to DNA replication.

Detailed studies on deletion mutants suggest that the histoné nascent polypeptide is not involved in the posttranscriptional regulatory process (Capasso et al., 1987). Systematic removal of approximately 100 nucleotide blocks spanning nearly the entire protein coding region had no effect on the destabilization of these mutant histone mRNAs when DNA synthesis was inhibited. Consistent with this result, the deletion of the ATG translation start codon which prevents the synthesis of histone protein did not prevent the destabilization of the mRNA during inhibition of DNA replication. Furthermore, as discussed above, the minimal target sequences containing the stem-loop structure confer instability to gamma globin mRNA when DNA synthesis is interrupted (Pandey and Marzluff, 1987). The globin-histone chimeric mRNA, although regulated as wild type histone mRNA, does not encode any histone protein sequences.

Destabilization of histone mRNA appears to occur in a stepwise manner initiating at the 3' terminus (Ross et al., 1986). The first detectable histone mRNA degradation product lacks 5 nucleotides from the 3' terminus (Ross and Kobs, 1986; Ross et al., 1986). This degradation product is detectable in the in vitro mRNA decay system as well as in

whole cells (Ross et al., 1986). It is unclear whether this product arises from a single endonucleolytic scission or from multiple cuts removing one or more of the nucleotides at a time. Degradation continues rapidly in a 3' to 5' direction carried out by an exonuclease which is part of the ribosome complex (Ross et al., 1987). These results are consistent with the requirement for translation to proceed near the 3' terminus for the destabilization of the histone mRNA during inhibition of DNA synthesis.

Subcellular Compartments

The appearance of histone mRNA on polysomes within minutes after transcription and the equally rapid transfer of the newly synthesized histone proteins into the nucleus where they complex with DNA imply that histone protein synthesis may be compartmentalized in a region located just outside the nucleus (Spaulding et al., 1966; Borun et al., 1967; Robbins and Borun, 1967). The asymmetric distribution of eukaryotic mRNAs in the cytoplasm supports this possibility. Morphological studies initially demonstrated that polyribosomes are concentrated in areas surrounding the nucleus in mouse 3T3 cells (Fulton et al., 1980). Later, localization of specific mRNAs by in situ hybridization techniques provided direct evidence that mRNAs are differentially distributed throughout the cell. Tubulin mRNA is preferentially located in the periphery of the cytoplasm

whereas vimentin mRNA is concentrated in perinuclear regions in chick myoblast and fibroblast cells (Lawrence and Singer, 1986). Furthermore, actin mRNA is concentrated in the lamellipodia, structures involved in cell locomotion (Lawrence and Singer, 1986). The requirement for actin protein in the lamellipodia during cell movement (Wang, 1984) suggests a functional relationship between where the mRNA is localized and where the encoded protein is needed (Lawrence and Singer, 1986).

Localization of mRNAs in distinct areas of the cytoplasm may occur due to an association of the mRNA with an underlying structure. This structure could serve as an anchor to concentrate mRNAs in particular regions of the cytoplasm. Consistent with this reasoning is the existence of a complex, proteinaceous structure in the cytoplasm of most eukaryotic cells that is referred to as the cytoskeleton. The cytoskeleton plays a role in cell shape, cell motility and the intracellular transport of macromolecules and organelles (Clarke and Spudich, 1977; Goldman et al., 1978; and reviewed in Nielsen et al., 1983). In addition, there is growing support for the hypothesis that the cytoskeleton plays a key role in regulating the translation process (Lenk et al., 1977; Lenk and Penman, 1979; Fulton et al., 1980; Cervera et al., 1981; van Verooij et al., 1981; Nielsen et al., 1983; Howe and Hershey, 1984).

The Cytoskeleton: Microtubules, Intermediate Filaments and Microfilaments

The cytoskeleton structure is an interconnected system of three major classes of protein fibers, namely the microtubules, intermediate filaments and microfilaments. Each system represents a heterogenous population of proteins. The microtubules, for example, are composed of 13 protofilaments of the well characterized alpha and beta tubulin proteins (Amos and Klug, 1974). These filaments are 25 nm in diameter and appear to contain a hollow core. Several microtubule associated proteins (MAPs) have also been identified which appear to facilitate the assembly of the microtubules and promote their interactions with other cellular organelles (Sloboda et al., 1976, Bulinski and Borisy, 1980; Bloom et al., 1984; Binder et al., 1985). It has long been recognized that in mitotic cells the microtubules reorganize into the mitotic spindle including the astral, kinetochore and continuous fibers which mediate the segregation of the chromosomes (Weber et al., 1975). During interphase the role of the microtubules is less clear and somewhat controversial. Studies on agents that cause the depolymerization of the microtubules, such as colchicine and colcemid, suggest that the microtubules play a role in cell shape and the intracellular transport of macromolecules and organelles (Hayden and Allen, 1984; Vale et al., 1985).

There are at least five major classes of protein that constitute the intermediate filaments: 1) desmin, 2) vimentin, 3) keratin, 4) neurofilaments and 5) glial filaments. Biochemical and immunological studies demonstrated that these proteins are generally expressed in a cell type specific manner (for review see Lazarides, 1980). Desmin is an acidic protein with a molecular weight of 53,000 Daltons and is predominantly found in intermediate filaments from cardiac, smooth and skeletal muscle cells (Lazarides and Hubbard, 1976; Hubbard and Lazarides, 1979). The localization of desmin at the Z line in muscle suggests a role for this protein in the coordination of the contractile actions of the muscle fiber (Lazarides and Hubbard, 1976; Izant and Lazarides, 1977).

Vimentin is found principally in intermediate filaments from cells of mesenchymal origin. Immunological and biochemical characterization of vimentin indicated that the protein is 52,000 Daltons and widely conserved throughout evolution (Brown et al., 1976; Bennett et al., 1978; Franke et al., 1979). A characteristic feature of the vimentin intermediate filaments is their sensitivity to colchicine which erroneously led researchers to believe initially that they were disaggregation products of the microtubules (Goldman and Knipe, 1973; Holltrop et al., 1974; Blose and Chacko, 1976). The collapse of vimentin into filamentous bundles around the nucleus during colchicine treatment

suggests that these filaments are in close association with the microtubules.

The keratin filaments, also known as tonofilaments, are predominantly found in epithelial cells. The keratin proteins are a family of approximately 30 distinct polypeptides ranging in molecular weight from 40,000-65,000 Daltons (Steinert, 1975; Steinert and Gullino, 1976; Sun and Green, 1978; Moll et al., 1982). The keratins can be clearly divided into acidic and basic groups based on two-dimensional electrophoretic analysis. Although only a few keratin subtypes are expressed in any one epithelial cell, there is always a coordinated synthesis of at least one class each of the acidic and basic keratin subunits (Moll et al., 1982; Woodcock-Mitchell et al., 1982; Fuchs and Marchuk, 1983). The combination of keratin subtypes expressed by an epithelial cell is characteristic of the tissue origin as well as the differentiation state of the cell (Roop et al., 1983; Sun et al., 1983). In addition, some epithelially derived tumors express different keratin subtypes than those found in the normal counterpart and these differences may be useful in diagnostic tests (Summerhayes et al., 1981; Osborn, 1983).

Little is known about the function and organization of the intermediate filaments from neuronal and glial cells. The major cytoskeletal element of neuronal axons and dendrites are the neuronal filaments which are generally

composed of three large polypeptides with molecular weights of 200,000, 150,000, and 68,000 Daltons (Hoffman and Lasek, 1975; Delacourte et al., 1980). Glial filaments are specifically found in astrocytes and other types of glial cells and consist of a single protein, the glial fibrillary acidic protein (GFA), with a molecular weight of 51,000 Daltons (Schachner et al., 1977; Goldman et al., 1978). Although the intermediate filament proteins from the different cell types are heterogeneous in their chemical composition, they assemble into similar morphological structures consisting of filaments that are 10 nm in diameter that are highly resistant to extraction with detergents. It has been proposed that the intermediate filaments contain a constant and variable region, analogous to the structure of antibodies. The constant domain would conserve the morphology whereas the variable domain would confer functional specificity (Lazarides, 1980).

The microfilaments are the thinnest of the cytoskeletal filament systems, with an average diameter of 6 nm, and are composed primarily of actin protein. It has been estimated that approximately 5-10% of total cellular protein is actin which exists in an equilibrium between monomeric and filamentous forms (Hartwig and Stossel, 1975). Microfilaments from non-muscle cells contain two predominant species of actin protein, namely beta- and gamma-actin. The non-muscle actin proteins are quite similar to muscle

specific alpha-actin with respect to molecular weight (42,000 Daltons), amino acid sequence and polymeric structure (Tilney and Detmers, 1975; Hartwig and Stossel, 1975; Kane, 1975; Garrels and Gibson, 1976; Whalen et al., 1976; Rubenstein and Spudich, 1977). These results suggest that béta- and gamma-actin protein in non-muscle cells may play a role in contraction processes such as cell motility , chromosome movement and exocytosis (Berl et al., 1973; Huxley, 1973; Sanger, 1975).

Cytoskeletal-mRNA Interactions

Early studies using stereo-electron microscopic analysis of whole cells indicated that polysomes are associated with the cytoskeleton structure (Wolosewick and Porter, 1977). Closer examination of the cell interior, by removing membranes and soluble cytoplasmic proteins with detergent, reveals that many of the ultrastructural features and nearly all the polysomes are retained on the cytoskeletal framework (Lenk et al., 1977). Furthermore, the non-random topological distribution of the polysomes is preserved in the cytoskeleton preparation (Fulton et al., 1980). The association of the polysomes with the cytoskeleton does not appear to be due to trapping since monosomes and independent ribosomal subunits are efficiently removed by the detergent extraction (Lenk et al., 1977; Cervera et al., 1981; Ornelles et al., 1986). These results

indicate that actively translated mRNAs are associated with the cytoskeleton and this association may be requisite for protein synthesis. Consistent with this possibility, the inhibition of host protein synthesis coincides with the release of host mRNA from the cytoskeletal structure and the attachment of viral mRNA to the cytoskeleton in poliovirus infected and adenovirus infected cells (Cervera et al., 1981; Lemieux and Beaud, 1982). During development, maternally inherited mRNAs participate in protein synthesis only at the time they become associated with the cytoskeleton in sea urchin oocytes (Moon et al., 1983).

The region of the polysome involved in its attachment to the cytoskeleton is not known. Previous results indicate that the association of polysomal mRNA with the cytoskeleton is through the mRNA or mRNP particle and not through the ribosome (Lenk et al., 1977; Lenk and Penman, 1979; Cervera et al., 1981; Lemieux and Beaud, 1982). Disruption of polysomes by heat shock, high salt, sodium fluoride or pactamycin treatment releases the ribosomal subunits but not the mRNA from the cytoskeleton into the soluble phase (Lenk et al., 1977; Cervera et al., 1981; van Venrooij et al., 1981; Howe and Hershey, 1984). Immunofluorescence studies indicate that the cap-binding protein is associated with the cytoskeleton suggesting that the attachment of eukaryotic mRNAs to the cytoskeleton may occur via the 5' mRNA cap structure (Zumbe et al., 1982). Earlier studies suggest that

the association of eukaryotic mRNAs with the cytoskeleton may occur through the poly-A tract (Milcarek and Penman, 1974). However, the association of uncapped poliovirus mRNAs (Bonneau et al., 1985) and poly-A$^-$ histone mRNAs (Jeffery, 1984; Bonneau et al., 1985) indicates that the attachment site might also exist in an internal region contained within the 5' and 3' ends of the mRNA. Isaacs and Fulton (1987) have presented evidence suggesting that myosin heavy chain in embryonic chicken muscle cells is co-translationally assembled into the cytoskeleton. The myosin nascent polypeptide would therefore serve as an additional cytoskeleton attachment site for the polysomal myosin mRNA. Based on these results it appears that several mechanisms may operate in the attachment of a specific polysomal mRNA or mRNP to the cytoskeleton and these attachment sites may vary depending on the species of mRNA.

The component of the cytoskeleton to which the mRNA is attached is not known although the data accumulated to date suggest that the microfilaments may be involved in the mRNA-cytoskeleton association. Removal of tubulin from the cytoskeleton by using cold calcium-containing buffers during the isolation procedure has no effect on the attachment of mRNA with the cytoskeleton; this result suggests that the microtubules do not play a role in the association of mRNA with the cytoskeleton (Lenk et al., 1977; Howe and Hershey, 1984; Ornelles et al., 1986). Disruption of vimentin

filaments with heat shock or indirectly with colcemid has no effect on protein synthesis as determined by two-dimensional electrophoresis indicating that translation is not dependent on the integrity of the intermediate filaments (Welch and Feramisco, 1985). Other reports suggest that the attachment of mRNA to the cytoskeleton is mediated by the microfilaments (Lenk et al., 1977; Howe and Hershey, 1984; Ornelles et al., 1986). Treatment with cytochalasins, either cytochalasin B or D, to disrupt the actin-containing microfilaments results in the release of poly-A^+ mRNA from the cytoskeleton into the soluble phase. At least in the case of cytochalasin D treatment, the release of poly-A^+ mRNA from the cytoskeleton occurs in a dose dependent manner and stoichiometrically reflects the degree of inhibition of protein synthesis (Ornelles et al., 1986). These results indicate that intact microfilaments are required for the association of mRNA to the cytoskeleton; however, it can not be concluded that the actin fibers directly bind the mRNA.

Nonmembrane-Bound and Membrane-Bound Polyribosomes: The Signal Hypothesis

As described above, nearly 100 percent of the actively translated polysomes are associated with the cytoskeleton. Studies on vesicular stomatitis virus infected HeLa cells demonstrated that membrane-bound polysomal mRNA coding for the viral G glycoprotein and "free" polysomal mRNA coding

for the polymerase, nucleocapsid and matrix proteins are all attached to the cytoskeleton during translation (Cervera et al., 1981). The association of free polysomes with the cytoskeleton has also been demonstrated by electron microscopy (Wolosewick and Porter, 1979). Free polysomes are generally the class of polysomes involved in the synthesis of intracellular proteins whereas membrane-bound polysomes are generally involved in the synthesis of exported proteins that are destined for the cell surface or secretion (for review see Emr et al., 1980). The association of free polysomal mRNAs with the cytoskeleton suggests that the polysome is anchored to a scaffold, albeit a dynamic structure, and not freely diffusible within the cytoplasm. The term "free polysome" is operationally defined and is a misnomer when viewed in light of the biochemical and microscopic studies on the cytoskeleton. The proper reference to free polysomes is nonmembrane-bound polysomes.

The mechanism by which mRNAs assemble into membrane-bound polysomes has been described in the signal hypothesis (Blobel and Dobberstein, 1975). Many types of signal peptides exist that target proteins to various subcellular membrane compartments such as the endoplasmic reticulum, mitochondria and chloroplasts in eukaryotes as well as the cell membrane in prokaryotes (Date et al., 1980; Schatz and Butow, 1983; Muller and Blobel, 1984; Cline, 1986). The work presented here is focused on events that occur at the

endoplasmic reticulum and on what is generally known for the synthesis of proteins that are localized on the cell surface or secreted from the cell. Most cell surface or secreted proteins are initially synthesized with a segment of amino acid residues at the N-terminus that is not usually found in the mature protein. This segment is commonly referred to as a signal peptide. Although signal peptides are not highly conserved in sequence, they are fairly conserved in structure and based on the compilation of 277 signal peptides ranging from bacteria to higher eukaryotes, a consensus structure has been deduced (Watson, 1984). In general, signal peptides are 25-30 amino acids in length. They contain a charged amino acid within the first five residues, which is followed by a stretch of at least nine hydrophobic amino acids, and terminate at a small uncharged amino acid such as alanine, serine or glycine. It is believed that the hydrophobic domain of the signal peptide facilitates the transfer of the protein across the lipid environment of specific intracellular membranes whereas the small, uncharged amino acid is thought to direct the protease to cleave the peptide from the mature protein (Hortin and Boime, 1981).

Presumably, translation of mRNA encoding exported proteins is initiated on nonmembrane-bound polysomes. Following the synthesis of approximately 70 amino acids, the point at which the entire signal peptide has emerged from

the ribosome, the signal recognition complex (SRP) binds to the signal peptide and arrests translation (Walter and Blobel, 1981). The SRP is an 11S ribonucleic acid-protein complex composed of six non-identical polypeptides and one small cytoplasmic RNA molecule (Walter and Blobel, 1980; Walter and Blobel, 1982; Walter and Blobel, 1983). The RNA molecule of SRP is 300 ribonucleotides in length and shares approximately 80% homology with a middle repetitive human DNA family (Ullu et al., 1982; Li et al., 1982). With the SRP bound to the signal peptide, the complex can then associate with the membrane of the endoplasmic reticulum through the interaction with the SRP receptor which is also known as the docking protein (Gilmore et al., 1982a; Gilmore et al., 1982b; Meyer et al., 1982a; Meyer et al., 1982b). The SRP then dissociates from the polysome with the resultant release of the translational block. As translation resumes the protein is co-translocated from the cytoplasm into the endoplasmic reticulum. During translocation, a signal peptidase which is located in the lumen of the endoplasmic reticulum removes the signal peptide from the protein (Maurer and Mckean, 1978; Walter et al., 1984).

Overview of Project

The overall aim of this work is to better define the mechanism(s) involved in coupling histone protein synthesis with DNA replication. Specifically, we have focused our

attention on the posttranscriptional regulation of histone gene expression. The evidence cited above suggests that the cytoplasm of eukaryotic cells is a highly organized region which contains subcellular compartments that concentrate translation factors as well as specific mRNAs (Fulton et al., 1980; Howe and Hershey, 1984; Lawrence and Singer, 1986; Ornelles et al., 1986). Traditionally, the idea of subcellular compartments refers to organelles such as the nucleus, endoplasmic reticulum, and mitochondria. For example, a popular biochemical definition of compartmentation is "a simple regulatory mechanism in which the enzymes for catabolism and biosynthesis are physically separated by membrane-bound cell organelles" (Rawn, 1983). A broader definition of subcellular compartmentation includes any specialized region of the cell regardless of the presence of a membrane and may be extended to such components and regions as 1) nonmembrane-bound and membrane-bound polysomes, 2) cytoskeleton and soluble phase, and 3) spatial location (i.e., near the nucleus versus near the plasma membrane).

Histone mRNAs and the factors that are involved in their selective degradation during the inhibition of DNA synthesis may be localized in a particular subcellular compartment. Consistent with this reasoning, the subcellular compartment may influence the posttranscriptional control of histone gene expression. Based on this possibility, we have

specifically asked the question: Does the subcellular location of histone mRNA play a role in the destabilization of histone mRNA during inhibition of DNA synthesis? To address this question, we have applied a combination of molecular and cellular approaches first, to determine the natural location of histone mRNA and second, to alter the subcellular compartment to study whether the foreign location affects histone mRNA stability. The rationale for this approach is that directing histone mRNA to an altered subcellular location may physically remove the message from the factors that are involved in the selective degradation of histone mRNA during inhibition of DNA synthesis.

Our initial studies demonstrated that histone mRNAs are translated predominantly on nonmembrane-bound polysomes that are associated with the cytoskeleton. The subcellular location of the mRNA was altered biologically by constructing a histone fusion gene that contains sequences coding for the signal peptide of the beta-lactamase gene of E. coli pBR322 plasmid DNA. The encoded fusion histone mRNA was directed to membrane-bound polysomes and was not destabilized during inhibition of DNA synthesis. Inactivation of signal peptide function by a single nucleotide substitution in the ATG translation start codon of the signal peptide resulted in the association of the mutated fusion mRNA with nonmembrane-bound polysomes and restored the coupling of the mRNA stability with DNA

replication. These results indicated that the subcellular location plays a role in the posttranscriptional control of histone gene regulation.

During the course of studying histone mRNA subcellular localization it was necessary to examine histone mRNA-cytoskéletal interactions in detail. Previously, Ornelles et al. (1986) reported that total poly-A^+ mRNA, as a heterogenous population, is released from the cytoskeleton in HeLa cells by cytochalasin D treatment. We demonstrated by Northern blot analysis that cytochalasin D treatment of HeLa cells readily releases histone mRNA, which is not poly-adenylated, from the cytoskeleton into the soluble phase. Surprisingly, mRNA coding for the cell surface HLA-B7 class I antigen remained associated with the cytoskeleton during cytochalasin D treatment. Subsequent studies demonstrated that HLA-B7 mRNA expresses at least two distinct cytoskeletal attachment sites: a cytochalasin D sensitive site and a puromycin sensitive site. The puromycin sensitive site appears to be a feature of membrane-bound polysomal mRNAs and is most likely the nascent polypeptide and/or ribosome associated with the protein substructure of the endoplasmic reticulum.

Cytochalasin D and puromycin cotreatment, conditions that efficiently release HLA-B7 membrane-bound polysomal mRNA and histone nonmembrane-bound polysomal mRNA from the cytoskeleton into the soluble phase, did not dissociate the

signal peptide-histone fusion mRNA from the cytoskeleton. The nucleotide sequences of the signal peptide-histone fusion mRNA encoding the signal peptide contain a cytochalasin D and puromycin insensitive cytoskeletal attachment site which is not expressed by histone mRNA or HLA-B7´mRNA. These results provide evidence for the heterogeneic pattern for cytoskeleton-mRNA interactions.

Lastly, we investigated the role of the cytoskeleton in the regulation of gene expression by studying the effects of disrupting the microfilaments with cytochalasin D on the transcription and steady state mRNA levels of several genes. We determined that the transcriptional and posttranscriptional regulation of the c-fos proto-oncogene is quite sensitive to the perturbation of the cytoskeleton structure. Transcription of the c-fos gene is selectively stimulated with a concomitant increase in c-fos mRNA steady state levels within minutes of the addition of cytochalasin D. Consistent with previous results, the transcription of beta-actin was also elevated in cytochalasin D treated cells. These findings suggests that the cell monitors the organization of the cytoskeleton and selectively regulates gene expression accordingly.

CHAPTER 2

GENERAL METHODS

Materials

[α-^{32}P]dCTP (~3,000 Ci/mmol), [α-^{32}P]dATP (~3,000 Ci/mmol), [gamma-^{32}P]ATP (~3,000 Ci/mmol) and [α-^{35}S]dATP (>1,000 Ci/mmol) radionucleotides were purchased from Amersham, Arlington Heights, IL; polyvinylsulfonic acid (PVS), polyethylene glycol 8,000 (PEG), XAR-5 x-ray film were obtained from Eastman Kodak Co., Rochester, NY; ultrapure electrophoresis grade agarose and Zeta-probe nylon membranes were from Bio Rad, Richmond, CA; formaldehyde solution (37% w/w) was from Fisher Scientific, Fair Lawn, NJ; nitrocellulose was from Schleicher and Schuell, Keene, NH; RPI scintillator ppo-popop concentrated liquid scintillator (Liquifluor) was from Research Products International, Elk Grove Village, IL; formamide was from Bethesda Reasearch laboratories (BRL), Gaithersburg, MD; acrylamide, calf intestine alkaline phosphatase, nuclease S1, E. coli DNA polymerase I large fragment (Klenow enzyme) and most restriction endonucleases were obtained from Boehringer Mannheim Biochemicals (BMB), Indianapolis, IN; T4 DNA ligase was from New England Bio Labs (NEB), Beverly, MA;

T4 polynucleotide kinase (cloned) was from United States Biochemical Corp. (USB), Cleveland, OH; cytochalasin D, polyoxyethylene sorbitan monopalmitate (Tween 40), sodium deoxycholate (NaDOC), sodium dodecyl sulfate (SDS), Triton X-100, and DNase I (DN-EP) were purchased from Sigma, St. Louis, MO; lysozyme was purchased from Worthington Biochemicals, Freehold, N.J.; fetal bovine, calf bovine and horse sera were obtained from Gibco Laboratories, Grand Island, NY or Flow Laboratories, McLean, VA; cycloheximide was kindly provided by Upjohn, Kalamazoo, Michigan.

Conditions for Enzyme Reactions

The buffers and incubation conditions for each of the restriction endonucleases and DNA modifying enzymes used in this study were as described by the specific recommendations of the manufacturers unless otherwise indicated.

Mammalian Cell Culture

HeLa Monolayer Cell Cultures

HeLa S3 suspension cells (human cervical carcinoma cell line; ATCC ccl 2.2) were seeded into EMEM (Eagles-modified minimum essential medium) containing 5% fetal calf serum, 5% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM glutamine at $3x10^6$ cells per 10 cm tissue culture dish and incubated at 37°C under 5% CO_2. Cells were maintained at sub-confluent densities by splitting the cultures 1:10 into fresh medium every 3 to 5 days.

HeLa Suspension Cell Cultures

HeLa S3 cells were grown and maintained in suspension at 3-6x10^5 cells/ml in SMEM (Joklik-modified minimum essential medium) supplemented with 7% calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM glutamine in a warm róom at 37°C. Monoclonal and polyclonal HeLa S3 cell lines, which were isolated during G418 selection of transfected monolayer cultures, were maintained in SMEM containing 5% fetal calf serum, 5% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM glutamine at 3-6x10^5 cells/ml.

WI-38 Monolayer Cell Cultures

WI-38 cells (normal, human diploid fibroblast-like cells; ATCC ccl 75) at passage 28, were grown as monolayers in EMEM containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM glutamine at 37°C under 5% CO_2. Cell cultures were made quiescent by maintaining cultures in EMEM containing 0.5% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM glutamine at 37°C under 5% CO_2 for 48 hours.

Metabolic Inhibitor Treatments

Inhibition of DNA synthesis was usually performed with 1-5 mM hydroxyurea for 30 to 60 minutes. The drug was always prepared fresh in 1X spinner salts (Gibco) as a 100 mM stock solution.

Protein synthesis was inhibited with 10 μg/ml cycloheximide for varying lengths of time. Cycloheximide stock solutions [500 μg/ml] were prepared fresh in 1X spinner salts. Protein synthesis was also inhibited with 0.4 mM puromycin for varying lengths of time. Puromycin stock solutións [54.4 mg/ml] were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C.

To study the dependence of protein synthesis on the destabilization of histone mRNA during the inhibition of DNA synthesis, we pre-treated cells with 10 μg/ml cycloheximide for 5 minutes and then added 1 mM hydroxyurea for an additional 30 minutes.

Disruption of the cytoskeleton was generally achieved by treating the cells with 5 to 60 μg/ml cytochalasin D for 15-20 minutes at 37°C. The cytochalasin D stock solutions [5-10 mg/ml] were prepared in DMSO and stored at -20°C.

Plasmid DNA

The human histone genes used in this study were previously isolated by Sierra *et al.* from a lambda Ch4A bacteriophage library (1982). The histone genes were subsequently subcloned into *Escherichia coli* pBR322 plasmid DNA and propagated in *E. coli* strain HB101 bacterium (Sierra *et al.*, 1982; Plumb *et al.*, 1983; Stein *et al.*, 1984). Recombinant plasmids ST519 and FF435C each contain a distinct genomic DNA fragment encoding a human H3 histone

gene. The recombinant plasmid FO108A contains an entire human H4 histone gene which includes the promoter, coding and 3' non-transcribed regions.

An HLA-B7 cDNA clone, pDP001, was generously supplied by Dr. Sherman Weissman, Yale University, New Haven, Connecticut (Sood et al., 1981). The pDP001 plasmid DNA was constructed from the human cell line RPMI 4265 and contains a 1400 bp insert coding for HLA-B7 in the PstI site of pBR322 plasmid DNA. Therefore, the plasmid confers tetracycline resistance and not ampicillin resistance to E. coli HB101 bacteria.

The phCGα plasmid DNA was generously provided by Dr. John Fiddes, California Biotechnology Inc., Mountain View, California and Dr. John Nilson, Case Western Reserve University, Cleveland, Ohio. The phCGα plasmid is a cDNA clone containing a 621 bp fragment coding for the alpha subunit of human chorionic gonadotropin inserted into the HindIII site of pBR322 and confers ampicillin resistance (Fiddes and Goodman, 1979).

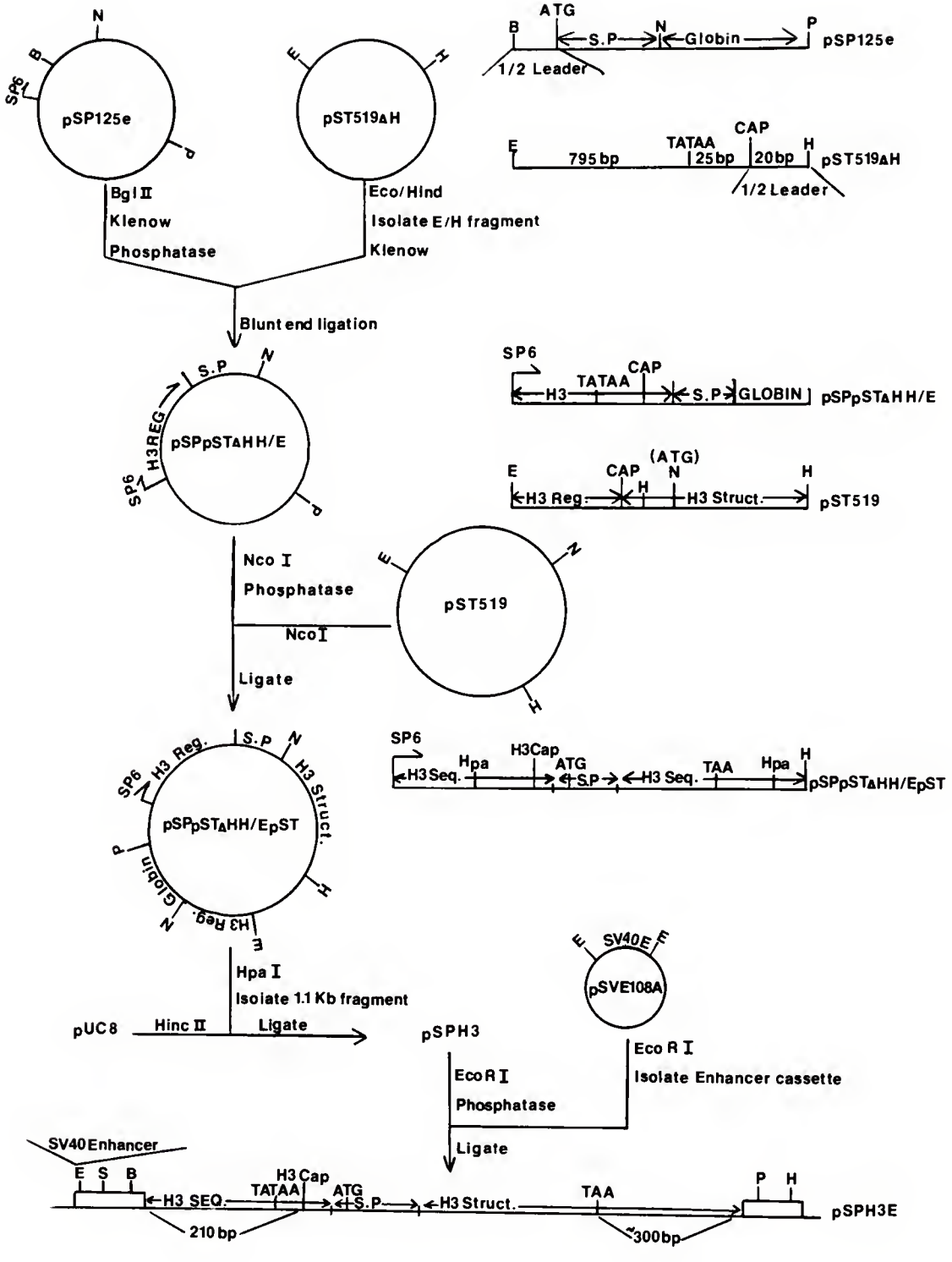
The beta-lactamase signal peptide-globin fusion gene, pSP125e, was kindly provided by Dr. Vishwanath Lingappa, University of California at San Francisco. The pSP125e clone contains the signal peptide from the beta-lactamase gene of E. coli plasmid pBR322 fused in frame to the coding sequence for chimpanzee alpha globin. This fusion gene was subcloned into the pSP64 vector (Promega Biotech) as a HindIII-PstI

fragment and is under control of the SP6 promoter (Simon et al., 1987).

To study the regulation of histone mRNA localized on membrane-bound polysomes, we constructed a beta-lactamase signal peptide-histone fusion gene as outlined in Figure 2-1. The first intermediate, pSPpSTdeltaHH/E, involved the placement of the E. coli pBR322 beta-lactamase signal peptide-globin fusion gene from pSP125e under transcriptional control of the cell cycle-dependent H3 histone regulatory region of pST519deltaH. The 840 bp EcoRI/HindIII fragment from pST519deltaH (Marashi et al., 1986) was isolated electrophoretically and blunt-ended using the Klenow fragment of DNA polymerase I. The signal peptide-globin fusion construct was digested with BglII restriction endonuclease, which cuts once in the untranslated leader region of the fusion gene, blunt-ended with Klenow fragment and dephosphorylated using calf intestinal alkaline phosphatase. The 840 bp blunt-ended fragment from pST519 was ligated into the BglII blunt-ended site of pSP125e using T4 DNA ligase. The construction of the second intermediate, pSPpSTdeltaHH/EpST, involved the substitution of the chimpanzee globin coding sequences in pSPpSTdeltaHH/E with the H3 histone coding sequences from pST519. The pSPpSTdeltaHH/E DNA was digested with NcoI, which cuts at the signal peptide-globin coding region junction, and treated with calf intestinal phosphatase. The pST519 DNA was

Figure 2-1. Outline of the cloning scheme for the construction of the signal peptide-histone fusion gene.

Restriction endonuclease sites are as follows: B, BglII; E, EcoRI; H, HindIII; N, NcoI; P, PstI; S, SmaI. Abbreviations: S.P., E. coli pBR322 beta-lactamase signal peptide coding sequences; H3REG, transcriptional H3 histone regulatory region including CAAT and TATAA consensus sequences; CAP, histone H3 mRNA transcription start site; H3 struct, sequences coding for H3 histone protein; ATG, translation start codon; TAA, translation stop codon; SP6, bacteriophage SP6 promoter.


pSP125e
pST519ΔH
Bgl II
Klenow
Phosphatase
Eco/Hind
Isolate E/H fragment
Klenow
Blunt end ligation
pSPpSTΔHH/E
Nco I
Phosphatase
Nco I
pST519
Ligate
pSPpSTΔHH/EpST
Hpa I
Isolate 1.1 Kb fragment
pUC8
Hinc II
Ligate
pSPH3
pSVE108A
Eco R I
Isolate Enhancer cassette
Eco R I
Phosphatase
Ligate
SV40 Enhancer
H3 SEQ.
H3 Cap
TATAA
ATG
S.P
H3 Struct.
TAA
210 bp
300bp
pSPH3E
pSP125e
1/2 Leader
795 bp
25bp
20bp
pST519ΔH
H3
GLOBIN
pSPpSTΔHH/E
H3 Reg.
H3 Struct.
pST519
H3 Seq.
Hpa
H3Cap
TAA
pSPpSTΔHH/EpST

digested with NcoI, which cuts at the ATG translation start codon, and ligated to NcoI/dephosphorylated pSPpSTdeltaHH/E DNA using T4 DNA ligase. The signal peptide-histone fusion gene was subcloned into pUC8 plasmid DNA by ligating the 1.1 kbp HpaI fragment from pSPpSTdeltaHH/EpST into the HincII site of pUC8 to form pSPH3. The signal peptide-histone fusion gene in pSPH3 was transcriptionally enhanced by placing the SV40 viral enhancer element from pSVE108A (Shiels et al., 1985) into the EcoRI site in the polylinker of pSPH3 to form pSPH3E1 (containing one enhancer element) and pSPH3E2 (containing 2-3 enhancer elements).

DNA Isolation and Purification

Plasmid DNA Preparations

Plasmid DNAs used in the experiments described here were harbored in Escherichia coli strain HB101 bacteria (F^-, recA 13) unless otherwise indicated. Bacteria containing the appropriate plasmid were grown in medium containing antibiotics (usually 25-50 μg/ml ampicillin or tetracycline) as specified by the genotype of the plasmid or bacterium.

The following procedure is the routine method for isolating pBR322 derived plasmid DNAs and includes an amplification step with chloramphenicol for higher yields of DNA (Maniatis et al., 1982a). Plasmids that are derived from pUC8 DNA are also prepared as follows; however, due to their high copy number the amplification step is unnecessary. The

standard protocol for the isolation of pUC8 related plasmid DNAs is the same as described below except that the 500 ml culture is inoculated directly and allowed to grow overnight into the late log stage.

Starter cultures were prepared by inoculating 5 ml LB medium (Luria-Bertani medium: 1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl) supplemented with 0.1% glucose with 25-50 ul of bacterial glycerol stock and incubated overnight at 37°C. The 5 ml starter culture was transferred to 500 ml pre-warmed LB medium supplemented with glucose and incubated at 37°C until the optical density at 590 nm ($O.D._{590}$) of the culture reached 0.55 units. Plasmid DNA was amplified by treating the culture with 200 μg/ml chloramphenicol (prepared as a 20 mg/ml stock solution in 95% ethanol and filter sterilized) at 37°C for 12-16 hours. The bacteria were centrifuged at 5,000 rpm, 4°C for 10 minutes in a Beckman JA-10 rotor and J2-21 centrifuge. The cell pellet was resuspended in 10 ml solution I (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA, 5 mg/ml lysozyme) and incubated at room temperature for 5 minutes. The cells were lysed at 0°C with 20 ml solution II (1% SDS, 0.5 M NaOH) for 5 minutes. The cell lysate was treated with 15 ml 5 M potassium acetate, pH 4.8 and kept on ice for 5 minutes. The lysate was transferred to a 50 ml polycarbonate tube and centrifuged at 16,000 rpm for 20 minutes at 4°C in Beckman JA-20 rotor to pellet the bacterial DNA and cell debris. The

supernatant was transferred to a 150 ml Corex tube (Corning) and nucleic acids were precipitated from solution with 30 ml isopropanol at room temperature for 15 minutes. The DNA was pelleted by centrifugation at 5,000 rpm in Beckman JS-7.5 rotor for 30 minutes at 20°C. The pellet was briefly dried under vacuum and resuspended in 8 ml 10:1 TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). The DNA solution was adjusted to a final concentration of 1 mg/ml of CsCl (refractive index of 1.3860) and 600 μg/ml ethidium bromide. The DNA solution was then transferred to a 16 x 76 mm Quick-Seal tube (Beckman) and centrifuged to equilibrium at 45,000 rpm in a Beckman Type-50 Ti rotor and Beckman L5-65 ultracentrifuge for 36-48 hours at 20°C. The supercoiled plasmid DNA was removed from the cesium chloride gradient by puncturing the side of the tube just below the DNA band with a syringe containing a 22 gauge needle. The DNA was extracted repeatedly with 1-butanol to remove the ethidium bromide and to reduce the volume of the solution. Cesium chloride was removed by dialysis against 10:1 TE buffer at 4°C. The DNA was quantitated by absorbance at 260 nm and stored at -20°C. Routinely, the yield for pBR322 derived plasmids was 500-800 μg DNA per 500 ml amplified culture.

M13 Bacteriophage DNA Preparations

M13 is a filamentous DNA bacteriophage that is specific for male E. coli bacteria. M13 phage DNA is useful for site

directed mutagenesis and dideoxy DNA sequencing reactions since it can be readily isolated as a double-stranded covalently closed circular DNA replicative form (RF) from the infected bacteria and as a circular, single-stranded DNA genome (SS) from the mature phage particle. In addition, M13 phage DNA has been altered genetically to facilitate cloning and subsequent manipulations. One such derivative, M13mp18, is small in size, maintained in high copy number (approximately 200 RF molecules per cell), contains a convenient polylinker for the insertion of foreign DNA (up to 25 kb), and expresses beta-galactosidase which is inactivated by the insertion of DNA into the polylinker site.

Single-stranded templates. Single-stranded M13 DNA templates were prepared from individual "plaques" which formed on a lawn of *E. coli* strain JM103 bacteria (delta(*lac*-*proAB*), supE / F'[*tra*D36, *pro*A^+, *pro*B^+, *lac*I^q, *lac*ZdeltaM15]) that were infected with M13 bacteriophage or transfected with M13 double stranded, covalently closed circular DNA. A single plaque was removed from the agar plate with a sterile pasteur pipette and placed in 2 ml YT medium (0.8% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl). Two drops of an overnight JM103 bacterial culture were added to the phage and incubated at 37°C with agitation for 6 hours. Longer incubation times result in deletions

within the phage DNA. The cell culture (1.5 ml) was transferred to a microcentrifuge tube and spun in an Eppendorf centrifuge at 12,000 x g for 10 minutes at 4°C. The supernatant (1.2 ml) was transferred to a sterile microcentrifuge tube, 300 ul of 20% PEG, 2.5 M NaCl were added and the sample was incubated at room temperature for 15 minutes. The remaining 200-300 ul of supernatant were removed and stored at -20°C as a phage stock. The phage suspension was centrifuged at 12,000 x g for 5 minutes at room temperature and the supernatant was decanted. The pellet was resuspended in 150 ul TES buffer (20 mM Tris-HCl pH 7.5, 10 mM NaCl, 0.1 mM Na_2EDTA). The sample was extracted with phenol as follows: 1) added 50 ul phenol and vortexed for 2 seconds; 2) incubated at room temperature for 5 minutes; 3) vortexed for 2 seconds; and centrifuged at 12,000 x g for 4 minutes at room temperature. DNA was precipitated from 130 ul of aqueous phase with 5 ul 3 M sodium acetate and 340 ul 95% ethanol on crushed dry ice for 15 minutes and collected by centrifugation at 12,000 x g, for 15 minutes at 4°C. The supernatant was discarded and the pellet was washed in 70% ethanol at room temperature. The sample was centrifuged at 12,000 x g, room temperature for 5 minutes. The DNA pellet was dried under vacuum, resuspended in 80 ul TES buffer and stored at -20°C.

Double-stranded templates. E. coli strain JM101 (delta(lac-proAB), supE / F' [proA$^+$, proB$^+$, lacI^q, lacZdeltaM15]) bacteria were grown overnight in 5 ml YT medium at 37°C with agitation. The overnight culture (1 ml) was transferred to 100 ml prewarmed YT medium and incubated at 37°C until the O.D.$_{590}$ reached 0.4 units. To assure exponential growth the culture was again diluted 1:100 into 500 ml prewarmed YT medium and grown to an O.D.$_{590}$ of 0.4 units. The culture was infected with 500 ul of M13 bacteriophage supernatant which had been prepared from an isolated plaque as described above for the preparation of M13 single-stranded DNA. The infected cells were incubated at 37°C with agitation for no more than 8 hours. The cells were harvested and RF M13 DNA was isolated as described above for the preparation of plasmid DNA. Routinely, the yield was 300 μg of supercoiled M13 DNA per 500 ml culture.

Isolation of Mammalian RNA

Isolation of Total Cellular RNA

The following procedure for the isolation of total cellular RNA was developed by Dr Mark Plumb in the Stein's laboratory primarily for work with HeLa cells. However, this procedure has been successfully used without modification for the isolation of total cellular RNA from human HL-60 cells, mouse 3T3-L1 cells, rat osteoblast primary cell cultures, and Trypanosome cruzii cells.

Exponentially growing HeLa cells (5 x 10^7 cells) were harvested by centrifugation at 1,500 rpm for 5 minutes at 37°C in an IEC rotor. The cells were washed in phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate pH 6.8) and resuspended in 3 ml lysis buffer (2 mM Tris-HCl pH 7.4, 1 mM EDTA) containing 5 μg/ml PVS. Cells were lysed in the presence of 2.4% SDS and 88 μg/ml of proteinase K at room temperature for 15 minutes. Lysates were adjusted to 300 mM NaCl, extracted with 5 ml Tris-saturated phenol and 5 ml $CHCl_3$/isoamyl alcohol (IAA) (24:1, v/v) and centrifuged at 3,000 rpm in an IEC rotor at room temperature. The aqueous phase was then extracted with 5 ml $CHCl_3$/IAA and centrifuged at 3,000 rpm in an IEC rotor at room temperature. The phenol/chloroform and chloroform extractions were repeated as described above. Nucleic acids were precipitated from the aqueous phase with 2.5 volumes of 95% ethanol at -20°C in the presence of 53 mM potassium acetate.

Nucleic acids were recovered from the ethanol suspension by centrifugation at 12,000 x g and resuspended in TCM buffer (10 mM Tris-Hcl, pH 7.4, $CaCl_2$, 10 mM $MgCl_2$) for digestion with DNase I. The DNase I was dissolved in 20 mM Tris-HCl pH 7.4, 10 mM $CaCl_2$ at 1 mg/ml, preincubated at 37°C for 20 minutes and treated with 0.1 volume of proteinase K [1 mg/ml] at 37°C for 2 hours to remove ribonuclease activity (Tullis and Rubin, 1980). The nucleic

acid samples were then digested with 0.1 mg/ml of proteinase K treated DNase I for 20-30 minutes at 37°C. Following DNase I digestion, 0.05 volumes of 5 M NaCl and 0.25 volumes of 10% SDS were added to each sample and the RNA solutions were repeatedly extracted with phenol and chloroform until the interface between the aqueous and organic phases was clean. RNA was precipitated from the aqueous phase with 2.5 volumes of 95% ethanol, at -20°C overnight. The RNA was collected at 12,000 rpm in a JA20 rotor at 4°C for 30 minutes. The pellet was resuspended in double distilled water, quantitated by optical density at 260 nm and stored at -20°C. Typical yields of RNA were 400 μg per 90% confluent 10 cm plate of HeLa S3 cells and 1 mg per 5 x 10^7 HeLa S3 cells with an $O.D._{260}/O.D._{280}$ of 1.8 to 2.0.

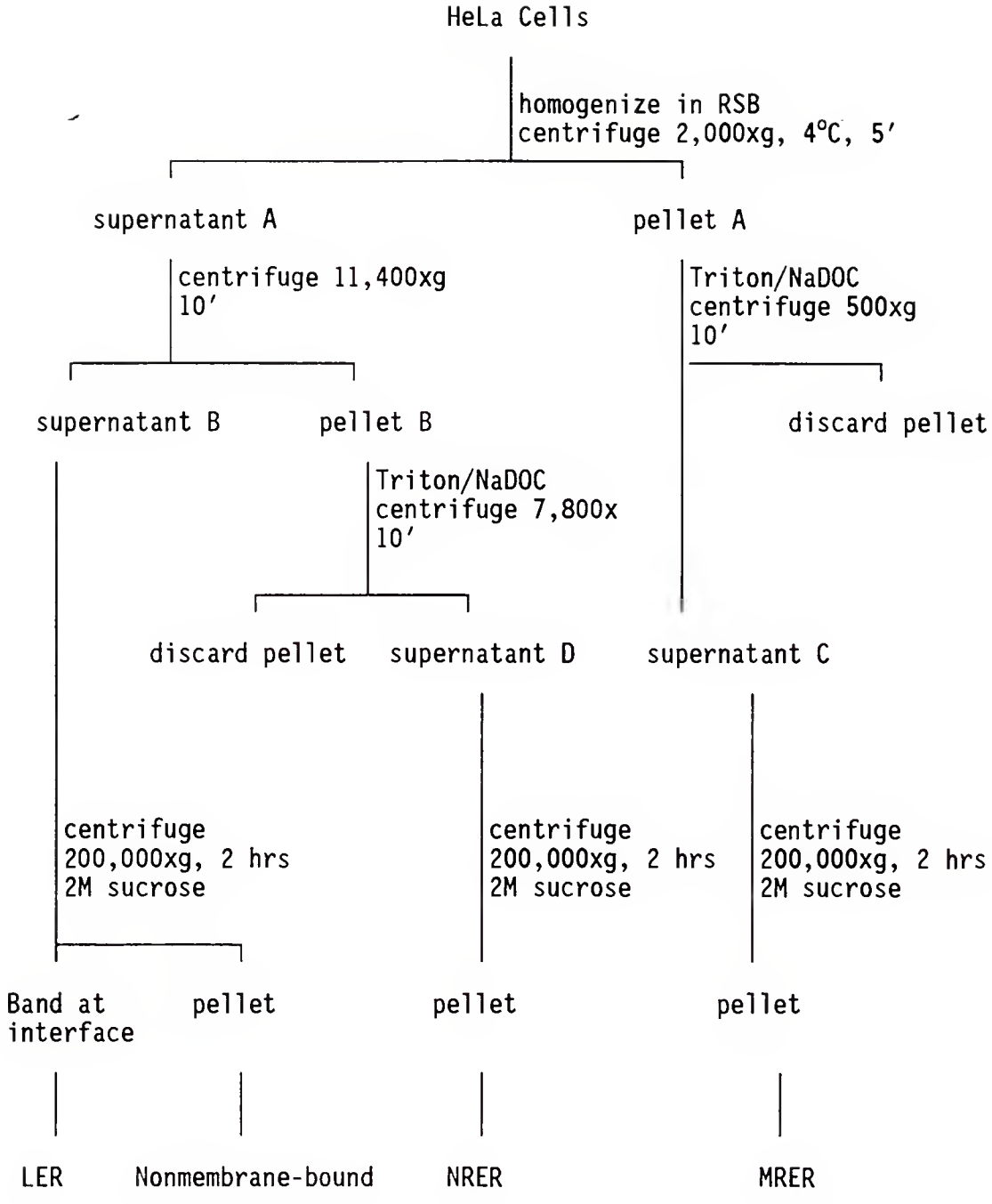
Isolation of Nonmembrane-bound, Membrane-bound and Total Polysomal RNA

The subcellular fractionation scheme used in these studies is a modified procedure described by Venkatesan and Steele (1972) and is outlined in Figure 2-2. Exponentially growing HeLa cells (5 x 10^8 cells) were collected by centrifugation at 1,500 rpm in an IEC rotor at 37°C for 5 minutes. All subsequent procedures were carried out at 4°C. Cells were washed several times in PBS and resuspended in 25 ml RSB (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 2.5 mM $MgCl_2$). After incubation on ice for 20 minutes, the cells were

disrupted with 15 strokes in a tight fitting Dounce homogenizer. The homogenate was then centrifuged at 2,000 x g for 5 minutes. The supernatant (supernatant A) was removed and maintained in an ice bath while the nuclear pellet (pellet A) was resuspended in 9 ml TMN (20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 25 mM NaCl, 3 mM dithiothreitol). The nuclear suspension (pellet A) was adjusted to 1% Triton X-100 and 1% NaDOC, vortexed briefly and centrifuged at 500 x g for 10 minutes. The supernatant (supernatant C) from this centrifugation step was removed and saved on ice for the isolation of nucleus-associated rough endoplasmic reticulum (NRER) polysomes. The supernatant from the centrifugation step following homogenization (supernatant A) was centrifuged at 12,000 rpm, 4°C for 10 minutes in a Beckman JA20 rotor. The resulting supernatant (supernatant B) was removed onto ice for subsequent isolation of light endoplasmic reticulum (LER)-associated polysomal RNA and nonmembrane-bound polysomal RNA. The pellet (pellet B) was resuspended in 9 ml TMN, adjusted to 1% Triton X-100 and 1% NaDOC and centrifuged at 10,000 rpm, 4°C for 10 minutes in a JA20 rotor. The supernatant (supernatant D) was removed and stored on ice for the isolation of mitochondria-associated rough endoplasmic reticulum (MRER) polysomal RNA.

Exponentially growing HeLa cells (2.5 x 10^8 cells) were collected by centrifugation for the isolation of total polysomal RNA. The cell pellet was washed in PBS and

Figure 2-2. Outline of procedure for the isolation of subcellular polysomal fractions.


HeLa Cells
homogenize in RSB
centrifuge 2,000xg, 4°C, 5′
supernatant A
pellet A
centrifuge 11,400xg
10′
Triton/NaDOC
centrifuge 500xg
10′
supernatant B
pellet B
discard pellet
Triton/NaDOC
centrifuge 7,800x
10′
discard pellet
supernatant D
supernatant C
centrifuge
200,000xg, 2 hrs
2M sucrose
centrifuge
200,000xg, 2 hrs
2M sucrose
centrifuge
200,000xg, 2 hrs
2M sucrose
Band at
interface
pellet
pellet
pellet
LER
Nonmembrane-bound
NRER
MRER

resuspended in 15 ml TMN. The sample was adjusted to 1% Triton X-100 and 1% NaDOC, disrupted with several strokes in a tight fitting Dounce homogenizer and centrifuged at 12,000 x g at 4°C for 10 minutes in a JA20 rotor. The supernatant was removed and saved at 0°C.

Each of the fractions (NRER, MRER, LER, nonmembrane-bound, and total polysomal RNA) were layered onto a 3 ml 2 M sucrose pad (prepared in RSB containing 100 uM spermidine) and centrifuged at 4°C for 2 hours at 48,000 x g in a Beckman Type 50.2 Ti rotor (200,000 x g) without the brake. The LER formed a cloudy band at the top of the sucrose pad and was carefully removed by aspiration. The remaining fractions were pelleted under these conditions and gently resuspended in 3 ml TMN buffer. The samples were then extracted extensively with phenol and chloroform as described above for the isolation of total cellular RNA. The RNA was precipitated from solution with 3 volumes of 95% ethanol at -20°C in the presence of 53 mM potassium acetate. The RNA was digested with DNase I, extracted with phenol and chloroform, and ethanol precipitated as described above for the preparation of total cellular RNA. Typical yields were 1.5-2.0 mg MRER and NRER RNA, 2-3 mg nonmembrane-bound RNA, 1 mg LER RNA and 4-6 mg total polysomal RNA per liter of exponentially growing HeLa cells.

Isolation of Cytoskeleton and Soluble Phase RNA

The cytoskeleton and soluble fractions were prepared as previously described by Penman and coworkers (Cervera et al., 1981). Exponentially growing HeLa cells (5 x 10^8 cells) were harvested and washed in cold PBS. The cell pellet was resuspended in 2 ml extraction buffer (10 mM Pipes pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 0.3 M sucrose, 1 mM phenylmethylsulfonyl fluoride) and Triton X-100 was immediately added to a final concentration of 0.5%. The sample was incubated at 0°C for 3 minutes and then centrifuged at 700 rpm in an IEC centrifuge for 3 minutes at 4°C. The supernatant (SOL) was removed and stored on ice. The pellet was resuspended in 8 ml RSB containing 1 mM phenylmethylsulfonyl fluoride, 1% Tween 40 and 0.5% NaDOC. The nuclei were stripped of cytoplasmic tags with 15 strokes in a precision-bore stainless steel homogenizer with a clearance of 0.002 inches and were pelleted at 2,000 rpm for 3 minutes at 4°C in an IEC rotor. The supernatant (CSK) was removed and saved at 0°C for subsequent RNA isolation. Both the CSK and SOL fractions were extracted in the presence of 1% SDS and 0.3 M NaCl with phenol and chloroform. Nucleic acids were precipitated from the aqueous phase with 3 volumes of 95% ethanol at -20°C in the presence of 53 mM potassium acetate. The RNA was digested with DNase I, phenol and chloroform extracted, and ethanol precipitated as described above for the isolation of total cellular RNA.

Typical yields of RNA were 250-300 μg from CSK and 100-150 μg from SOL per 2.5 x 10^7 HeLa cells (50 ml exponentially growing suspension culture).

Two-Dimensional Gel Electrophoresis and Immunoblotting Analysis

The cytoskeleton and soluble fractions were isolated as described above and each sample was dialyzed in 2 mM Tris-HCl pH 7.5 and treated with DNase I (20 μg/ml) and RNase A (20 μg/ml) in the presence of 0.5 mM $MgCl_2$ at 4°C for 1 hour. The samples were dialyzed against 2 mM Tris-HCl pH 7.5 and then lyophilized. Two-dimensional gel electrophoresis and immunoblotting analyses were performed by Dr. Warren Schmidt (Department of Pathology, Vanderbilt University, Nashville, TN). The protein preparations were focused in the first dimension using equilibrium conditions as described by O' Farrell (1975) with pH 3 to 10 or 5 to 7 ampholines. The second dimension was electrophoresed on slab gels containing a 3% stacking gel and 7.5% resolving gel as described by Laemmli (1970). The proteins were then either stained with Coomassie Brilliant Blue or electrotransferred to nitrocellulose filters as described by Towbin et al. (1979). Where indicated, immunotransfer analyses (Glass et al., 1981) were performed with commercially available or other (Schmidt et al., 1982) antibodies to actin, tubulin and prekeratin.

Northern Blot Analysis

Agarose-Formaldehyde RNA Denaturing Gel Electrophoresis

RNA samples were resolved electrophoretically in 1.5% agarose-6% (w/v) formaldehyde horizontal slab gels and running buffer (20 mM MOPS (morpholinepropane-sulfonic acid) pH 7.0, 5 mM sodium acetate, 1 mM EDTA, 3.7% (w/v) formaldehyde) as described by Rave et al. (1979). Routinely 10 μg RNA per sample were dried in a Savant Speed Vac concentrator and resuspended in 3.2 ul double distilled water (ddH_2O), 5 ul formamide, 1 ul 10x MOPS (200 mM MOPS pH 7.0, 50 mM sodium acetate, 10 mM EDTA), 0.8 ul 37% (w/v) formaldehyde and 2 ul sample buffer (0.5 ml formamide, 0.1 ml 10x MOPS, 80 ul 37% (w/v) formaldehyde and 0.32 ml 0.2% bromophenol blue-90% glycerol). The samples were then heated at 60°C for 10 minutes, quick chilled to 0°C and loaded onto the gels. The 1.5% agarose-6% formaldehyde gels were prepared by melting 3 g electrophoresis grade agarose in 144 ml ddH_2O plus 20 ml 10x MOPS buffer in a microwave oven (Whirlpool) at full power for 3-4 minutes. The agarose solution was cooled to approximately 65°C (warm to the touch) while stirring and then adjusted to 6% formaldehyde with 36 ml 37% (w/v) formaldehyde. For minigels, 35 ml agarose-formaldehyde solution was cast in a plexiglass gel tray (10 cm x 6.3 cm) with an 8 well comb (1 mm x 5 mm per well). The gel was electrophoresed at 40-45 milliamps until the bromophenol blue migrated 9 cm (approximately 1.5

hours). For full size RNA gels, 200 ml agarose-formaldehyde solution was cast in a plexiglass gel tray (24.5 cm x 19.8 cm) with 2: 20 well combs (1 mm x 6 mm per well). The gel was electrophoresed at 100-120 volts until the bromophenol blue migrated 11 cm (approximately 4 hours). The formaldehyde gels were stained for 20 minutes in ethidium bromide solution (1 μg/ml ethidium bromide, 0.1 M ammonium acetate) and destained for at least 2 hours in double distilled water. RNA samples were then visualized by fluorescence under ultraviolet light using a short wavelength transilluminator (UVP, inc.; San Gabriel, CA). The gels were usually photographed at f4.5 for 1/4 second with Type 57 high speed Polaroid film and a Polaroid model 545 camera mounted on a stand above the transilluminator.

Transfer of RNA from Agarose-Formaldehyde Gels to Hybridization Filters

RNA samples were transferred to nitrocellulose (0.45 um) or nylon membrane hybridization filters in 20x SSC as previously described by Thomas (1975). The gels were soaked in 20x SSC (3 M NaCl, 0.3 M sodium citrate pH 7.0) for 20 minutes and were not stained with ethidium bromide. The hybridization filters were uniformly wetted in ddH_2O and then 20x SSC. The gel was placed with the wells facing downward onto a sheet of 3 MM paper (Whatman) and sponge which had been soaked in 20x SSC. Strips of used X-ray films

were placed on areas of the sponge that were not covered by the gel so as to direct the flow of 20x SSC only through the gel. The filter was directly layered onto the gel and any air bubbles that were trapped between the gel and filter were carefully removed. The transfer set-up was completed by placing two sheets of 3 MM paper followed by 2-3 boxes of facial tissues (Kleenex brand) on top of the hybridization filter. The buffer chamber was filled with 20x SSC and transfer was carried out for 36 hours (note: complete transfer is usually achieved within 14 hours). After transfer, the filters were rinsed in 2x SSC for 10 minutes to remove particles of agarose, air dried and baked *in vacuo* at 80°C for 2 hours. The filters were stored in sealed hybridization bags at 4°C.

Hybridization of Filters-Immobilized RNA (Northern Blot)

Filters (nylon or nitrocellulose) were prehybridized for 3-4 hours in 0.5 ml hybridization buffer per cm^2 filter area at 43°C for the detection of chorionic gonadotropin alpha, HLA-B7 and c-fos mRNA and at 48°C for histone mRNA. Hybridization buffer consisted of 50% formamide, 5x SSC, 10x Denhardt's (100x Denhardt's: 2% (w/v) ficoll 400, 2% (w/v) polyvinylpyrrolidone), 1% SDS, 50 mM sodium phosphate pH 7.0, 20 μg/ml BSA, and 250 μg/ml *E. coli* DNA. The filters were hybridized at the appropriate temperature for 36 hours in 0.1 ml hybridization buffer per cm^2 filter containing

$1x10^6$ cpm/ml of each thermally denatured, ^{32}P-radiolabeled DNA probe. After hybridization, the filters were washed with 5x SSC, 1x Denhardt's (0.5-1 ml/cm^2 filter) for 10-15 minutes at room temperature to remove unbound probe. The filters were then washed, in order, with the following solutions (0.5 ml/cm^2 filter area): 1) 5x SSC, 1x Denhardt's; 2) 2x SSC, 0.1% SDS; 3) 1x SSC, 0.1% SDS; and 4) 0.1x SSC, 0.1% SDS at 60°-65°C for 30 minutes per wash. After washing the filters were briefly air dried and then exposed to preflashed Kodak XAR5 x-ray film at -70°C with a Cronex Lightning Plus intensifying screen (Dupont) for varying lengths of time. Hybridization was quantitated by scanning laser densitometric analysis of multiple exposures of autoradiographs that were within the linear range of the film.

S1 Nuclease Protection Analysis

S1 nuclease protection analysis was performed essentially as described by Berk and Sharp (1977) and was used in the work presented here to detect simultaneously both endogenous H3 histone mRNA and signal peptide-histone fusion mRNA in the same RNA sample. The S1 probe was prepared by digesting 30 μg pSPH3E1, pSPH3E1alpha, or pSPH3E1ATG$^-$ DNA with 30 units of SmaI at 30°C for 3 hours. The 450 bp SmaI fragment was isolated from a 0.8% agarose minigel by the freeze-squeeze method. The solution

containing the SmaI fragment was butanol extracted to reduce the volume and to remove the ethidium bromide. The fragment was then extracted with phenol and chloroform and precipitated in 2.5 volumes 95% ethanol at -20°C overnight. The DNA was collected by centrifugation at 12,000 x g at 4°C for 15 minutes and resuspended in 5 ul 10 mM Tris-HCl pH 8.0, 5 ul 10x CIP buffer (0.5 M Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine) and 38 ul ddH_2O. The blunt-ended DNA was dephosphorylated with 0.12 units calf intestinal alkaline phosphatase (CIP) at 37°C for 15 minutes and then at 55°C for 15 minutes. A second aliquot of CIP was added and the incubations at 37° and 55°C were repeated. The reaction was terminated with 40 ul ddH_2O, 10 ul 10x STE (100 mM Tris-HCl pH 8.0, 1 M NaCl, 10 mM EDTA) and 5 ul 10% SDS and heated at 68°C for 15 minutes. The sample was extracted with phenol and chloroform, adjusted to 0.25 M sodium acetate and precipitated with 2.5 volumes of 95% ethanol at -20°C overnight. The dephosphorylated SmaI fragment was pelleted at 12,000 x g, 4°C for 15 minutes, vacuum dried and resuspended in 12 ul 1x kinase buffer (66 mM Tris-HCl pH 9.5, 10 mM $MgCl_2$, 10 mM beta-mercaptoethanol, 2 mM spermidine). The S1 fragment was phosphorylated with 50 uCi [gamma-^{32}P]ATP and 1 ul polynucleotide kinase [30 units/ul] at 37°C for 30 minutes. The reaction was terminated with 0.5 ul 100 mM EDTA at 70°C for 10 minutes. The DNA solution was diluted with 1 ml elutip low salt buffer (20 mM Tris-HCl pH 7.4, 0.2 M NaCl, 1 mM EDTA) and passed over a Schleicher and

Schuell Elutip column. The column was washed with 5 ml low salt buffer to remove unincorporated radiolabeled ribonucleotide and the DNA fragment was eluted in 400 ul high salt buffer (Tris-HCl pH 7.4, 1 M NaCl, 1 mM EDTA). The radiolabeled S1 probe was usually used immediately and any excess was stored in high salt buffer at 4°C.

Routinely, 10-25 μg RNA (30 ul) and 10 ul S1 probe were co-precipitated with 100 ul 95% ethanol at -20°C overnight. The sample was centrifuged at 12,000 x g, 4°C for 15-30 minutes. The pellet was vacuum dried and resuspended in 10 ul 5x hybridization buffer (0.2 M Pipes pH 6.4, 2 M NaCl, 5 mM EDTA) and 40 ul recrystallized formamide. The sample was heated at 90°C for 10 minutes and then incubated at 55°C for 3 hours. Single-stranded nucleic acids were digested with S1 nuclease by adding 400 ul ice cold S1 nuclease buffer (0.03 M sodium acetate pH 4.6, 0.25 M NaCl, 1 mM $ZnSO_4$) and 900 units S1 nuclease at 37°C for 30 minutes. The S1 digestion reaction was terminated by organic extraction with 0.5 volume phenol and 0.5 volume chloroform and then 0.5 volume chloroform alone. Nucleic acids were precipitated from the aqueous phase with 1 ml 95% ethanol at -20°C overnight.

The S1 nuclease digested samples were collected at 12,000 x g, 4°C for 30 minutes. The pellets were vacuum dried and resuspended in 5 ul loading buffer (80% formamide, 1x TBE [10x TBE: 500 mM Tris-HCl pH 8.3, 500 mM boric acid, 10 mM EDTA], 0.1% bromophenol blue, 0.1% xylene cyanol). The

samples were denatured by heating at 100°C for 2 minutes and then quickly chilled at 0°C to prevent reannealing prior to loading onto the gel. Six percent polyacrylamide-8.3 M urea (cross linked at 20:1 acrylamide:bis-acrylamide) gels with dimensions of either 16 cm x 18 cm x 1.5 mm (1.5 mm x 6 mm 15 well comb or 1.5 mm x 4 mm 20 well comb) or 32.5 cm x 44 cm (0.5 mm x 8 mm 25 well comb) were prepared and pre-electrophoresed for 30 minutes. The samples were loaded with drawn-out capillary tubes or a Rainin Pipetteman (0-20 ul capacity) using flat pipette tips. The large gels were electrophoresed at 50 watts constant power (~1200 V, ~45 A) for 3-4 hours and the small gels were electrophoresed at 300 V (25-35 A) for 2-3 hours. The gels were placed on 2 sheets of 3 MM Whatman paper, dried under vacuum at 80°C for 1-2 hours and exposed to pre-flashed XAR5 x-ray film with a Cronex Lightning Plus intensifying screen at -70°C for varying lengths of time. S1 analysis was quantitated by scanning laser densitometry of multiple autoradiographic exposures that were within the linear range of the film.

In Vitro Nuclear Run-On Transcription Analysis

The *in vitro* nuclear run-on transcription assays were performed by Anna Ramsey-Ewing as described by Baumbach *et al.* (1987). Cells were harvested by centrifugation and the cell pellet washed twice in cold isotonic buffer (125 mM KCl, 30 mM Tris-HCl pH 7.9, 5 mM $MgCl_2$, 10 mM beta-

mercaptoethanol). Cells were disrupted by homogenization (6-12 strokes) with a Dounce homogenizer, Wheaton type A pestle. After >90% of the cells had been lysed, nuclei were pelleted by centrifugation at 2000 rpm for 10 minutes in an IEC centrifuge at 4°C and resuspended in nuclei storage buffer containing 40% glycerol (50 mM Tris-HCl pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA). Nuclei were aliquoted and either snap frozen in liquid nitrogen or used fresh in the in vitro transcription reactions. Reactions typically contained 10^7 nuclei, 100 uCi alpha-^{32}P-UTP (3000 Ci/mmole), 1 mM ATP, 0.25 mM GTP and CTP in a final volume of 130 ul and were incubated for 30 minutes with intermittent shaking at 30°C. Radiolabeled RNAs were isolated by treatment of nuclei with DNase I (100 μg/ml) in the presence of 0.6 M NaCl, 50 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$ for 15 minutes at room temperature. The mixture was then incubated with proteinase K (200 μg/ml) for 30-60 minutes at 37°C in the presence of 150 mM NaCl, 12.5 mM EDTA, 100 mM Tris-HCl pH 7.5 and 20 mM $MgCl_2$. Sodium acetate (pH 5.5) was added to 0.2 M and nucleic acids extracted several times by the hot phenol method (Clayton and Darnell, Jr., 1983; Sherrer and Darnell, 1962). To the aqueous solution of ^{32}P-labeled RNAs, 150 μg of yeast RNA and 2.5 volumes of 95% ethanol were added. Precipitation was overnight at -20°C. Radiolabeled transcripts were resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA and an aliquot of each sample was precipitated with 150

μg yeast RNA and cold 10% TCA. TCA-precipitable counts were determined by liquid scintillation spectrometry.

DNA excess hybridization has also been described by Baumbach et al. (1987). In a preliminary experiment we determined that 2 μg of H4 histone insert in pFO002 was at least á two-fold DNA excess when hybridized to in vitro nuclear run-on transcripts. Southern blots containing restriction endonuclease digested DNA fragments or slot blots containing linearized plasmid DNAs were prepared. Southern blots on nitrocellulose or slot blots on Nylon were prehybridized in 1 M NaCl, 20 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.1% SDS, 5x Denhardt's, 250 μg/ml E. coli DNA and 12.5 mM sodium pyrophosphate at 65°C for at least 6 hours. Hybridizations were conducted at 65°C for 72 hours in 1 M NaCl, 20 mM Tris-HCl pH 7.4, 2 mM EDTA, 0.1% SDS, 2.5x Denhardt's, 250 μg/ml E. coli DNA with ^{32}P-labeled transcripts at $5x10^5$-$1X10^6$ TCA-precipitable counts per ml of hybridization solution. Blots were washed at 65°C for 15 minutes in fresh prehybridization solution [0.13-0.14 ml/cm^2], 1 hour in 2x SSC/0.1% SDS [0.5 ml/cm^2], overnight in 2x SSC/0.1% SDS [0.5 ml/cm^2] and 1 hour in 0.2x SSC/0.1% SDS [0.5 ml/cm^2]. Air dried filters were autoradiographed with XAR-5 or Cronex film and Cronex lightning Plus screens at -70°C for varying periods of time and developed with an X-O-MAT X-ray film processor.

Radiolabeling DNA for Northern Blot Analysis

DNA used for Northern blot analysis was uniformally labeled by nick translation as described by Maniatis et al. (1982b) or by the random priming method of Roberts and Wilson (1985). Radiolabeled probes prepared in this manner are also suitable for other purposes such as Southern blot analysis.

Random Priming Method

This protocol is very efficient for labeling small to intermediate-sized DNA fragments (100-2000 bp in length) and is the preferred method for work with purified DNA inserts. Supercoiled plasmid DNA is labeled much less efficiently by the random priming method and should be fragmented by sonication prior to use. Routinely, specific activities of $1x10^9$ cpm/μg DNA are achieved using as little as 20-100 ng DNA. Material for oligolabeling DNA fragments was purchased from Bethesda Research Laboratories as a kit.

The isotope (50 uCi [α-^{32}P] dCTP) was vacuum dried and resuspended in 10 ul of 2.5x reaction buffer (500 mM Hepes pH 6.6, 12.5 mM $MgCl_2$, 25 mM beta-mercaptoethanol, 125 mM Tris-HCl pH 8, 1 mg/ml BSA, 7.5 mg/ml synthetic DNA oligodeoxynucleotide primers, 50 uM each dGTP, dATP, dTTP). The DNA fragment (100 ng) in 14 ul ddH_2O was heated at 100°C for 2 minutes and immediately chilled on ice. The denatured DNA was added to the 1.5 ml microcentrifuge tube containing

the isotope and buffer and the priming reaction was initiated with 5 units Klenow enzyme [5 U/ul]. The sample was incubated at room temperature for at least 2 hours (usually overnight). The reaction was terminated with 1 ul 250 mM EDTA and 175 ul TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) and extracted once with 200 ul chloroform/isoamyl alcohol. Unincorporated radiolabeled deoxynucleotide was removed from the DNA by chromatography over a (10 mm x 100 mm) Biogel A-15m (Bio Rad) column in TE buffer. Fourteen fractions were collected; 800 ul in the first fraction and 200 ul in each subsequent fraction. Fractions containing radiolabeled DNA were determined by Cerenkov counting in the tritium channel with a Beckman LS-230 scintillation counter. The peak fractions were pooled and accurately quantitated by counting 2 ul DNA in 5 ml Triton-toluene based scintillation cocktail (2000 ml toluene, 1066 ml Triton X-100, 134 ml liquifluor) in the ^{32}P channel. The samples were immediately used for hybridization studies and excess probe was stored at 4°C.

Nick-Translation Method

Nick-translation was the common procedure for uniformally labeling DNA fragments or supercoiled DNA before the development of the random primer method. A disadvantage to using the nick-translation procedure is that the system must be optimized for efficiency for each lot of E. coli DNA

polymerase I enzyme. In addition, the specific activity (1-2 x 10^8 cpm/μg DNA) achieved with the nick-translation procedure is much lower than the random primer method (1 x 10^9 cpm/μg DNA).

DNase I [1 mg/ml in 10 mM HCl] was activated by incubating 1 ul of the enzyme in 9 ul DNase buffer (10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mg/ml BSA) at 0°C for 2 hours. The isotope (40-80 uCi [α-^{32}P] dCTP) was dried in a Savant Speed Vac centrifuge. The following components were added, in order, to the isotope : 1) 12.25 ul ddH_2O; 2) 2.5 ul 10x nick-translation buffer (500 mM Tris-HCl pH 7.8, 100 mM beta-mercaptoethanol, 50 mM $MgCl_2$); 3) 5 ul 10x dNTPs (100 uM of each dATP, dGTP, dTTP); 4) 1.25 ul BSA [1 mg/ml]; 5) 2.5 ul DNA [100 ng/ul]; and 6) 0.5 ul DNA polymerase I [5 U/ul]. The activated DNase I was diluted with 990 ul ice cold ddH_2O (1:1,000 dilution; 1 ng/ul final concentration) and 1 ul of diluted enzyme was added to the nick-translation mix. The sample was incubated at 14°C for 45 minutes and the reaction was then terminated with 20 ul 250 mM EDTA and 155 ul TE buffer. The sample was extracted once with 200 ul chloroform and unincorporated radiolabeled deoxyribonucleotides were removed by chromatography over a Biogel A-15m column. The DNA was quantitated and immediately used for hybridization.

Site-Directed Mutagenesis

In the past, researchers had to rely on nature to produce the proper mutations to study how the normal biological processes function. Zoller and Smith (1983) have designed a powerful method for selectively introducing single nucleotide mutations into specific sites of any cloned, sequenced region of DNA (site-directed mutagenesis). In addition, this procedure can be used to selectively create deletion or insertion mutations in DNA fragments of known sequence. The general approach of site-directed mutagenesis is to incorporate an oligonucleotide containing the appropriate mutation into the complementary strand of recombinant M13 DNA. The heteroduplex is transfected into bacteria and the resulting mutated bacteriophage are isolated by various selection processes.

As discussed in Chapter 4, the signal peptide-histone fusion mRNA is associated with membrane-bound polysomes and is stable during inhibition of DNA synthesis. It was unclear whether the uncoupling of the histone fusion mRNA stability from DNA replication was due to the altered subcellular location or to a change in mRNA structure, due to the nucleotide sequence coding for the signal peptide. To address these possibilities, we have introduced point mutations into the signal peptide coding region with the intention to inactivate signal peptide function. In doing so, mRNA structure is perturbed as little as possible and

the mutated histone fusion mRNA, like endogenous histone mRNA, associates with nonmembrane-bound polysomes.

Subcloning of the Signal Peptide-Histone Fusion Gene into M13 DNA

The signal peptide-histone fusion gene (SPH3) was subcloned into M13mp18 RF DNA. SPH3 DNA was digested with EcoRI and HindIII and the 1100 bp fragment was isolated from a 0.8% agarose gel. The M13mp18 DNA was digested with EcoRI and HindIII and the 7196 bp fragment was isolated from a 0.8% agarose gel. The 1100 bp SPH3 DNA fragment (~50 ng) and 7196 bp M13 DNA fragment (~50 ng) were ligated in 10 ul 1x ligation buffer (50 mM Tris pH 7.6, 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP and 50 μg/ml BSA) with 0.004 U T_4 DNA ligase (NEB) at 22°C overnight. The ligation mix was transfected into competent JM101 bacteria, white plaques were isolated, and M13/SPH3 recombinants were screened by restriction endonuclease digestion analysis. Single-stranded M13/SPH3 DNA was isolated from bacteriophage particles as described above.

Synthesis and Processing of Oligonucleotides

The signal peptide encoded by the SPH3 histone fusion gene was mutated by substituting leucine at amino acid position 10 and proline at position 12 with histidine residues. It was predicted that the introduction of two positively charged amino acids into the hydrophobic region

of the signal peptide would disrupt its recognition by the signal peptide recognition particle and would therefore, prevent the translocation of the mutated histone fusion mRNA to membrane-bound polysomes.

An oligonucleotide (SPα) containing two point mutations was synthesized by Tom Doyle in the Department of Microbiology and Immunology, University of Florida, Gainesville, Florida. The oligonucleotide was composed of the following sequence: 5' CAAAAAAGTGAATATGGGCGA 3', with the mutated nucleotides underlined. The oligonucleotide was received in ~2.5 ml 37% ammonium hydroxide and was heated at 55°C for 6 hours to remove the protecting amine group. The sample was dried under a gentle stream of air in the fume hood and resuspended in 1 ml ddH_2O. The sample was extracted two times with 600 ul n-butanol and the oligonucleotide was precipitated from the aqueous phase by adding 200 ul 0.5 M ammonium acetate and 2 ml 95% ethanol and chilling at -70°C for 30 minutes. The sample was centrifuged at 12,000 x g at 4°C for 30 minutes, washed in 80% ethanol and resuspended in 200 ul ddH_2O. The yield of oligonucleotide was estimated spectrophotometrically and was found to be 2.64 mg (A_{260}/A_{280} of 2.0).

A second oligonucleotide (ATG^-) was designed to destroy the signal peptide ATG translation start codon of the SPH3 fusion gene and thereby completely block the synthesis of any signal peptide. The $SPH3ATG^-$ mRNA retains the histone

translation start codon in its proper context, although further downstream from the 5' terminus than endogenous H3 histone mRNA, and should therefore be translated on nonmembrane-bound polysomes. In addition, SPH3ATG$^-$ mRNA differs from SPH3 wild type signal peptide-histone fusion mRNA at only one nucleotide. The sequence of the ATG$^-$ oligonucleotide is as follows: 5' AATACTCAAACTCTTCC 3', and was prepared and processed as described for the SPα oligonucleotide. The yield of ATG$^-$ oligonucleotide was only 445 μg (A_{260}/A_{280} of 1.41).

Optimization of Conditions for Site-Directed Mutagenesis

It was necessary to optimize the conditions for the hybridization of the oligonucleotide to M13 template so that the synthesis of the second strand was primed by the oligonucleotide from the correct site. Proper priming by the oligonucleotide was tested by primer extension analysis. After limited primer extension, the samples were digested with a restriction endonuclease that recognized a site downstream from the expected priming site. The number and intensity of the fragments produced by the digestion indicated the number of priming sites and the location of the preferred site.

The oligonucleotide (20 pmol) was phosphorylated with 5 units T_4 polynucleotide kinase in 100 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 5 mM dithiothreitol, 20 uCi [gamma-^{32}P] ATP at 37°C

for 45 minutes. The reaction was terminated by heating at 65°C for 10 minutes, and unincorporated radiolabeled nucleotides were removed by chromatography through a Sephadex G-25 (Pharmacia) column (10 mm x 100 mm) in 50 mM ammonium bicarbonate (pH 7.8).

The phosphorylated oligonucleotide (10-30 molar excess over template DNA) was added to 0.5 pmol of M13 recombinant DNA and the volume was adjusted to 10 ul with ddH_2O and 1 ul solution A (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl, 10 mM dithiothreitol). The solution was heated at 55°C for 5 minutes and then cooled for 5 minutes at room temperature. The primer extension reaction also contained 1 ul of 2.5 mM dCTP, 1 ul 2.5 mM dATP, 1 ul 2.5 mM dTTP, 1 ul 2.5 mM dGTP, 0.5 ul solution A and 1 unit Klenow enzyme. The reaction proceeded at room temperature for 5 minutes and was terminated by heating at 65°C for 10 minutes. The sample was diluted with 2 ul 10x PstI buffer (100 mM Tris-HCl pH 7.5, 1 M NaCl, 100 mM $MgCl_2$, 1 mg/ml BSA) and 7 ul ddH_2O and then digested with 1 ul PstI restriction endonuclease [5 U/ul] at 37°C for 1 hour. The sample was heated at 100°C for 2 minutes, quickly cooled to 0°C and diluted with 20 ul S1 loading buffer. The PstI digested samples (10 ul) were resolved electrophoretically in 5% acrylamide (20:1; acrylamide:bis), 7 M urea at 300 volts (~35 mA) for 1.5 hours. The gels were dried under vacuum at 80°C for 1 hour and analyzed by autoradiography. Primer extension analysis

revealed a single major band at ~80 bp for each molar ratio of oligonucleotide to template DNA studied. This was the expected size fragment and suggested that under these conditions, the oligonucleotide was priming the synthesis of the second strand from the proper site. If several predominant bands had been detected (i.e. multiple priming sites), more stringent annealing conditions would have been required.

Synthesis of the Mutated Strand

The oligonucleotide (1.3 μg) was phosphorylated in 100 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 5 mM dithiothreitol, 10 uM ATP with 15 units T_4 polynucleotide kinase at 37°C for 45 minutes. The reaction was terminated by heating at 65°C for 10 minutes.

The phosphorylated oligonucleotide (13.3 pmole) was annealed to M13/SPH3 recombinant DNA in 1x solution A by incubating the sample at 55°C for 5 minutes and then at room temperature for 5 minutes. After hybridization, the DNA was diluted with 10 ul solution C (1 ul solution B [0.2 M Tris-HCl pH 7.5, 0.1 M $MgCl_2$, 0.1 M dithiothreitol], 1 ul 10 mM dCTP, 1 ul 10 mM dTTP, 1 ul 10 mM dGTP, 0.5 ul 0.1 mM dATP, 1 ul 10 mM ATP, 1.5 ul [gamma-^{32}P] dATP, 1.5 ul T_4 DNA ligase [1 U/ul], 2 ul ddH_2O) and the synthesis of the second strand was initiated with 0.5 ul Klenow enzyme [100 U/16.7 ul] at room temperature. After 5 minutes, 1 ul 10 mM dATP was added

and the incubation was continued at room temperature overnight.

Enrichment for Covalently Closed Double-Stranded DNA

The *in vitro* synthesis of the entire second strand is very inefficient due primarily to the large size of the M13 genome. The percent of covalently closed, double-stranded DNA molecules is therefore, very small in comparison to the partially double-stranded, incomplete DNA molecules. These incomplete DNA molecules, when transfected into bacteria, give rise to wild type plaque formation which complicates the selection process for the mutated clones. Enrichment for covalently closed, double-stranded DNA molecules by alkaline sucrose gradient centrifugation greatly facilitates the selection process.

The DNA was precipitated from the overnight extension and ligation reaction by adding 30 ul ddH_2O, 50 ul 1.6 M NaCl-13% PEG at 0°C for 15 minutes. The DNA was pelleted at 12,000 x g at room temperature for 15 minutes. The pellet was gently washed in 100 ul cold 0.8 M NaCl-6.5% PEG, centrifuged at 12,000 x g for 30 seconds and resuspended in 180 ul TE buffer. The sample was denatured with 20 ul 2 N sodium hydroxide and layered onto a 5%-20% alkaline sucrose step gradient. The gradient was prepared by adding to a 0.5 in x 2 in centrifuge tube (nitrocellulose or polyallomer; do not use Beckman Ultra-clear tubes which are incompatible

with the alkali conditions), in order, the following solutions each containing 0.2 N NaOH, 1 M NaCl, 2 mM EDTA: 1) 1 ml 20% sucrose; 2) 1 ml 17.5% sucrose; 3) 1 ml 15% sucrose; 4) 1 ml 10% sucrose; 5) 5% sucrose. The tube was incubated at 4°C for 2 hours. The samples were centrifuged through the alkaline sucrose gradients in a Beckman SW 50.1 rotor at 37,000 rpm for 2 hours at 4°C without the brake. The samples were recovered from the gradients by puncturing the bottom of the tube with a 21 gauge needle and collecting 7 drops per fraction in 1.5 ml microcentrifuge tubes (usually ~60 fractions). Fractions containing covalently closed, double-stranded DNA were determined by Cherenkov scintillation counting in the tritium channel, pooled and neutralized with 1M Tris-citrate pH 5.

Isolation of Mutated M13 Recombinant Bacteriophage

The DNA was transfected into E. coli JM101 competent cells, plated onto YT agar and incubated overnight at 37°C. Individual plaques were picked and bacteriophage were subsequently prepared. Bacteriophage suspensions (2 ul) were spotted onto a 0.45 micron nitrocellulose filter and prehybridized in 6x SSC, 10x Denhardt's, 0.2% SDS (10 ml/100 cm^2) at 67°C for 1 hour. The filter was rinsed in 50 ml 6x SSC for 1 minute at 21°C and hybridized in 10 ml 6x SSC, 10x Denhardt's containing $1x10^6$ cpm radiolabeled oligonucleotide at 21°C for 1 hour. The filter was washed three times in 50 ml 6x SSC at 21°C for a total of 10 minutes. The filter was

briefly dried under a heat lamp and placed against pre-flashed XAR5 x-ray film without a screen for 1 hour at room temperature. Under such low stringency each phage spot was positive, including the negative control M13/SPH3 wild type bacteriophage. The filter was re-washed at 35°C in 6x SSC for 5 minutes, air dried and exposed to pre-flashed XAR5 film for 1 hour. At the higher wash temperature many of the phage spots were less intense whereas several remained unchanged. When the filter was washed at 48°C in 6x SSC for 5 minutes the negative control and nearly all the phage spots completely disappeared. The bacteriophage (~5%) that remained positive after the 48°C wash were sequenced to confirm the presence of the mutations.

DNA Sequencing

The site directed mutations in M13/SPH3 recombinant DNAs were confirmed by Sanger's dideoxynucleotide chain terminator DNA sequencing procedure using the "Sequenase™ DNA Sequencing Kit" from United States Biochemical Corporation, Cleveland, Ohio. The Sequenase enzyme is derived from bacteriophage T_7 DNA polymerase and has been modified to efficiently use dideoxyribonucleotides, alpha-thio deoxyribonucleotides and other nucleotide analogs that are commonly used for sequencing. In addition, the Sequenase enzyme expresses high processivity and low 3'-5' exonuclease activity.

Annealing Template and Primer

The primer for synthesizing the complementary strand of wild type and mutated M13/SPH3 recombinant DNA is a 20 bp oligonucleotide which hybridizes to a site adjacent to the polylinker. The annealing reaction was prepared by adding 1 ul primer (0.5 pmol), 7 ul single-stranded M13/SPH3 recombinant DNA (0.5-1.0 pmol) and 2 ul 5X sequencing buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl). The sample was heated at 65°C for 2 minutes and slowly cooled to room temperature.

Labeling Reaction

The complementary strand was synthesized by incubating the annealed sample with 1 ul 100 mM dithiothreitol, 2 ul labeling nucleotide mix (1.5 uM dGTP, 1.5 uM dCTP, 1.5 uM dTTP), 0.5 ul [α-^{35}S] dATP [10 uCi/ul] and 2 ul Sequenase [1.5 U/ul] at room temperature for 5 minutes. Elongation was terminated by mixing 3.5 ul aliquots of the labeling reaction with 2.5 ul of one of the dideoxy nucleotide solution (ddATP, ddCTP, ddGTP, ddTTP). The dideoxy nucleotide solutions were 80 uM dATP, 80 uM dCTP, 80 uM dGTP, 80 uM dTTP, 50 mM NaCl and 8 uM of the appropriate dideoxyribonucleotide. The sequencing samples were diluted with 4 ul stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), heated at 75°C for 2 minutes and loaded onto a 6% acrylamide-8.3 M urea gel

(32.5 cm x 44 cm) with a 60 well shark toothed comb. The gel was run at 75 W constant, ~2500 V, ~40 mA for 3-5 hours (Xylene Cyanol dye was 15 cm from the bottom of gel). The gel was soaked in 10% acetic acid-12% methanol in the fume hood for 1 hour, placed onto 2 sheets of 3 MM Whatman paper, and dried under vacuum at 80°C for 45 minutes. The gel was exposed to preflashed XAR5 x-ray film at room temperature for 12-18 hours.

Transfection of DNA into Cells

Introduction of Plasmid DNA into Bacterial Cells

An E. coli overnight cell culture was diluted 1:100 into 100 ml LB medium and incubated at 37°C until the optical density was approximately 0.37 at 590 nm. The cells were chilled at 0°C for 10 minutes and then centrifuged at 5,000 rpm, 4°C for 5 minutes in a Beckman JA20 rotor. The pellet was resuspended in 20 ml of cold $CaCl_2$ buffer (60 mM $CaCl_2$, 10 mM Pipes pH 7.0, 15% glycerol) and incubated at 0°C for 30 minutes. The cells were collected by centrifugation at 2,500 rpm for 5 minutes at 4°C in a JA20 rotor, resuspended in 2.6 ml $CaCl_2$ buffer and divided into 100-200 ul aliquots. The competent cells were either used immediately for transformation or stored in microcentrifuge tubes at -80°C.

For transformation, 100 ul competent E. coli cells were mixed with 10 ul plasmid DNA (~10 ng) and incubated at 0°C for 10 minutes. The cells were then heat shocked at 37°C for

5 minutes, diluted with 0.9 ml prewarmed LB medium and incubated at 37°C for 1 hour. Different volumes of the transfected bacterial cell culture (usually 10-200 ul) were plated onto the appropriate medium dictated by the genotype of the host and the introduced plasmid DNA.

Introduction of DNA into HeLa Cells

HeLa monolayer cell cultures were transfected with plasmid DNA, according to Gorman et al. (1982), in a calcium phosphate/DNA complex prepared as described by Graham and van der Eb (1973). HeLa S3 cells growing exponentially in suspension culture were plated into 20 ml completed EMEM (EMEM supplemented with 5% fetal calf serum, 5% horse serum, 100 U penicillin, 100 μg/ml streptomycin and 1 mM glutamine) at $3x10^6$ cells per 10 cm tissue culture dish (Corning or Falcon) and incubated overnight at 37°C in 5% CO_2. The monolayer cell culture was refed with 10 ml completed EMEM and incubated at 37°C in 5% CO_2 for 4 hours. The DNA/calcium phosphate precipitate was prepared by adding 20 μg DNA in 500 ul 250 mM $CaCl_2$ dropwise to 500 ul 2x Hebs buffer (50 mM Hepes, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.12±0.05) while vortexing the solution. The DNA/$CaCl_2$ precipitate was added dropwise to the monolayer cell culture and incubation was continued at 37°C in 5% CO_2 for 4 hours. The HeLa cells were glycerol shocked by replacing the medium with 2 ml completed EMEM containing 15% glycerol for 1 minute. The transfected

cells were then washed with 10 ml EMEM, refed with 20 ml completed EMEM and incubated at 37°C, 5% CO_2.

Establishment of Polyclonal and Monoclonal Stably Transformed Cell Lines

Polyclonal and monoclonal cell lines were isolated by growing HeLa cell cultures that were co-transfected with pSV2neo and the plasmid DNA of interest in medium containing the antibiotic G418. The plasmid pSV2-neo, constructed by Southern and Berg (1982), carries the bacterial aminoglycoside phosphotransferase 3' (II) gene under the control of the SV40 early promoter and confers resistance to the aminoglycoside antibiotic G418. The rationale behind the co-transfection method is that HeLa cells that are competent to take up pSV2-neo DNA are equally competent to take up the plasmid DNA of interest, and therefore, a certain percentage of the G418 resistant cells contain both species of DNA. Ideally, the selectable marker should be carried by the vector that contains the gene which is to be studied.

HeLa cells in monolayer culture were co-transfected with the plasmid DNA to be studied and pSV2-neo DNA (molar ratio of 20:1, respectively) by the calcium phosphate precipitation method as described above. After 36-48 hours post-glycerol shock, the transfected cells were resuspended in 5 ml Puck saline A [0.04% (w/v) KCl, 0.8% (w/v) NaCl, 0.035% (w/v) $NaHCO_3$, 0.1% (w/v) glucose] containing 0.02%

(w/v) EDTA and 0.5 ml of the cell suspension was seeded into 20 ml completed EMEM containing 500 μg/ml biologically active Geneticin (G418, Gibco) in 10 cm tissue culture dishes. The cells were incubated at 37°C, 5% CO_2 and refed every 3-4 days with completed EMEM containing G418 antibiótic. Within 2-3 weeks, the majority of the cells had died and individual colonies of G418 resistant HeLa cells had formed. Polyclonal cell lines were established by combining the G418 resistant colonies from a 10 cm dish as a single, heterogeneous cell population (usually 20-40 colonies). For the establishment of monoclonal cell lines, individual colonies were picked from the dish with sterile pasteur pipettes and cultured as separate cell lines.

CHAPTER 3

SUBCELLULAR LOCATION OF HISTONE mRNAs ON CYTOSKELETON ASSOCIATED NONMEMBRANE-BOUND POLYSOMES IN HELA S_3 CELLS

INTRODUCTION

Histone biosynthesis occurs primarily during the S phase of the cell cycle and is temporally as well as functionally coupled with DNA replication (Allfrey et al., 1963; Prescott, 1966; Spaulding et al., 1966; Robbins and Borun, 1967; Stein and Borun, 1972). Early studies using in vitro translation analysis of polysomal RNA isolated from S phase HeLa cells provided an initial indication that histone protein synthesis occurs on non-membrane-bound, light polysomes (Jacobs-Lorena et al., 1972; Liautard and Jeanteur, 1979; Gallwitz and Breindl, 1972; Borun et al., 1967). In these studies it was demonstrated that translatable, polysome-associated histone mRNAs have a sedimentation coefficient of 8-10S and fluctuate in a cell cycle-dependent manner. Histone mRNA was not detected by in vitro translation studies on polyribosomes isolated from HeLa cells in the G1 phase of the cell cycle (Borun et al., 1967; Borun et al., 1975; Pederson and Robbins, 1970). Consistent with this observation, the inhibition of DNA replication by hydroxyurea or cytosine arabinoside treatment

resulted in the rapid disappearance of histone mRNAs from these polysomes (Borun et al., 1967; Breindl and Gallwitz, 1974; Gallwitz, 1975).

The light polysomes on which translatable histone mRNAs reside are presumably nonmembrane-bound ("free") polysomes as predicted by the signal hypothesis (Blobel and Dobberstein, 1975). Several lines of evidence suggest that nonmembrane-bound polysomes are not freely diffusible within the cytoplasm but are attached to the cytoskeleton. Penman and co-workers (Lenk et al., 1977) have demonstrated that nearly 100% of total cytoplasmic polysomes co-fractionate with the cytoskeletal framework. In addition, vesicular stomatitis virus encoded nonmembrane-bound and membrane-bound polysomal mRNAs are associated with the cytoskeleton in virally infected HeLa cells (Cervera et al., 1983).

Taken together, these observations provide a basis for the possibility that histone mRNA-containing polysomes and/or their subcellular location may play a role in the regulation of histone gene expression. Such reasoning is consistent with the appearance of translatable histone mRNAs on polysomes within minutes following transcription (Borun et al., 1967), the equally rapid transfer of newly synthesized histones into the nucleus where they complex with DNA (Robbins and Borun, 1967; Spaulding et al., 1966), and the degradation of histone mRNAs and cessation of histone protein synthesis in parallel with the inhibition of

DNA replication (Spaulding et al., 1966; Robbins and Borun, 1967; Borun et al., 1967; Baumbach et al., 1984). To address the relationship between the localization of histone mRNAs and the control of cellular histone mRNA levels, we have used cloned human histone genes to examine the distribution of histone mRNAs on nonmembrane-bound and membrane-bound polysomes and the association of histone mRNA-containing polysomes with the cytoskeleton. As predicted from previous results of in vitro translation analysis and cDNA hybridization studies, histone mRNAs were found to reside predominantly on nonmembrane-bound polysomes. Both the nonmembrane-bound polysomes containing histone mRNAs and the membrane-bound polysomes containing the cell surface class I HLA-B7 antigen mRNAs were found associated with the cytoskeleton.

RESULTS

Localization of Histone mRNA and HLA-B7 mRNA in Subcellular Fractions

To determine the intracellular locations of histone mRNA and HLA-B7 mRNA, HeLa cells were first fractionated into nonmembrane-bound and membrane-bound polysomal components by the procedure outlined in Fig. 2-2. The membrane-bound polysomes are represented by the mitochondria associated rough endoplasmic reticulum (MRER) and the nucleus associated rough endoplasmic reticulum (NRER)

fractions. The light endoplasmic reticulum (LER), which is deficient in polysomes by definition, was also isolated following this subcellular fractionation scheme. Histone and HLA-B7 mRNA content in each of the subcellular fractions was determined by Northern blot analysis. Briefly, RNA was extracted from the samples, quantitated and electrophoresed in agarose under denaturing conditions. The RNA was then transferred to nitrocellulose filters for hybridization with ^{32}P-labeled (nick-translated) pFO108A (H4 histone), pFF435C (H3 histone) and pDP001 (cDNA clone of HLA-B7 class I antigen) DNA probes. A typical screening of subcellular fractions with these probes is represented by the autoradiograph in Figure 3-1, and Table 3-1 summarizes the numerical values derived from densitometric analysis of the Northern blot. The HLA-B7 mRNA and histone mRNA species are each present in total cellular RNA and total polysomal RNA which were included as positive controls. Non-membrane-bound polysomal RNA contains approximately 55% and 51% of the total H4 and H3 histone mRNA, respectively. Surprisingly, the LER fraction contains approximately 38% of the H4 histone mRNA and 41% of the H3 histone mRNA, which may be a consequence of the method by which the fraction is isolated. The LER and non-membrane-bound polysomal sample is centrifuged through a 2 M sucrose pad under conditions where the LER forms a loose band on top of the pad, while the nonmembrane-bound polysomes are pelleted. The LER band is

removed by aspiration and occasionally material is removed from within the sucrose pad. This material may be the source of histone mRNA in the LER fraction, which could decrease the true percentage of total histone message found on the nonmembrane-bound polysomes. The HLA-B7 mRNA was located almost exclusively on membrane-bound polysomes. Nearly 90% of the HLA-B7 mRNA was collectively found on the NRER and MRER, while less than 11% was measured in the LER and nonmembrane-bound polysomal RNA fractions.

Histone mRNA and HLA-B7 mRNA Distribution Between Csk and Sol Fractions

Existing within the cytoplasm of eukaryotic cells is an intricate network of protein filaments, which is referred to as the cytoskeleton. This structure can be prepared from HeLa cells treated with non-ionic detergent as described by Penman and coworkers (Cervera et al., 1981). We used this procedure for the isolation of the cytoskeleton (Csk) and soluble (Sol) fractions which were subsequently assayed for mRNA content by Northern blot analysis.

Log phase, suspension grown HeLa S_3 cells were fractionated into a Csk phase and Sol phase as described in Chapter 2. Although this fractionation procedure has been characterized previously (Lenk and Penman, 1979), we examined the protein heterogeneity of each fraction by two-dimensional gel electrophoresis and immunoblotting techniques in collaboration with Dr. Warren Schmidt. As seen

Figure 3-1. Northern blot analysis of H3 histone, H4 histone and HLA-B7 mRNA in subcellular fractions.

The cytoskeleton and soluble fractions, as well as the nonmembrane-bound and membrane-bound polysomal RNA fractions, were isolated from HeLa cells by the procedures described in Chapter 2 (Cervera et al., 1981; Venkatesan and Steele, 1972). In addition, total polysomal RNA and total cellular RNA were isolated (Plumb et al., 1983, Venkatesan and Steele, 1972). RNA was extracted from each subcellular fraction and 50 μg of RNA from each preparation was resolved by 1.5% agarose, 6% formaldehyde gel electrophoresis. The RNA was transferred to nitrocellulose and hybridized to radiolabeled pFF435C (H3 histone and pFO108A (H4 histone) probes, followed by hybridization to ^{32}P-labeled (nick-translated) pDP001 (cDNA of HLA-B7) probe. The hybridized filters were then exposed to preflashed XAR5 x-ray film at -70°C for 72 hours. From left to right, lanes: 1) total cellular RNA; 2) Csk RNA; 3) Sol phase RNA; 4) NRER; 5) MRER; 6) nonmembrane-bound polysomal RNA; 7) LER; and 8) total polysomal RNA.

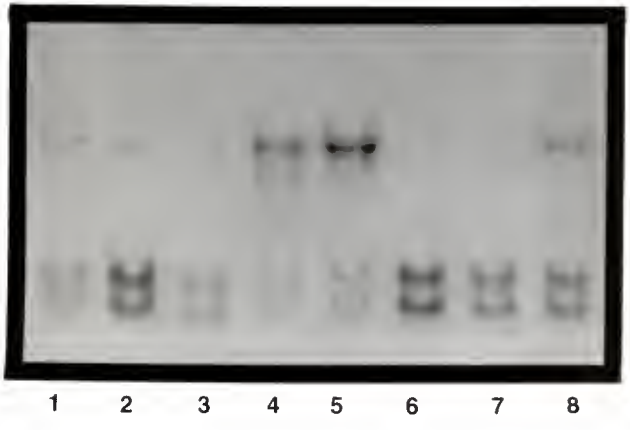

HLA-B7
H3
H4
1
2
3
4
5
6
7
8

Table 3-1. Quantity of histone and HLA-B7 mRNAs in the subcellular fractions.

	H3	H4	HLA-B7
Cytoskeleton	90%	84%	97%
Soluble	10%	16%	3%
NRER	3%	3%	29%
MRER	6%	5%	60%
Free	51%	54%	5%
LER	40%	38%	6%

The distribution of histone and HLA-B7 mRNAs in the NRER, MRER, LER and nonmembrane-bound polysomal fractions as well as the cytoskeleton and soluble fractions was determined by Northern blot analysis. The values have been normalized with respect to the total yield of RNA within each fraction.

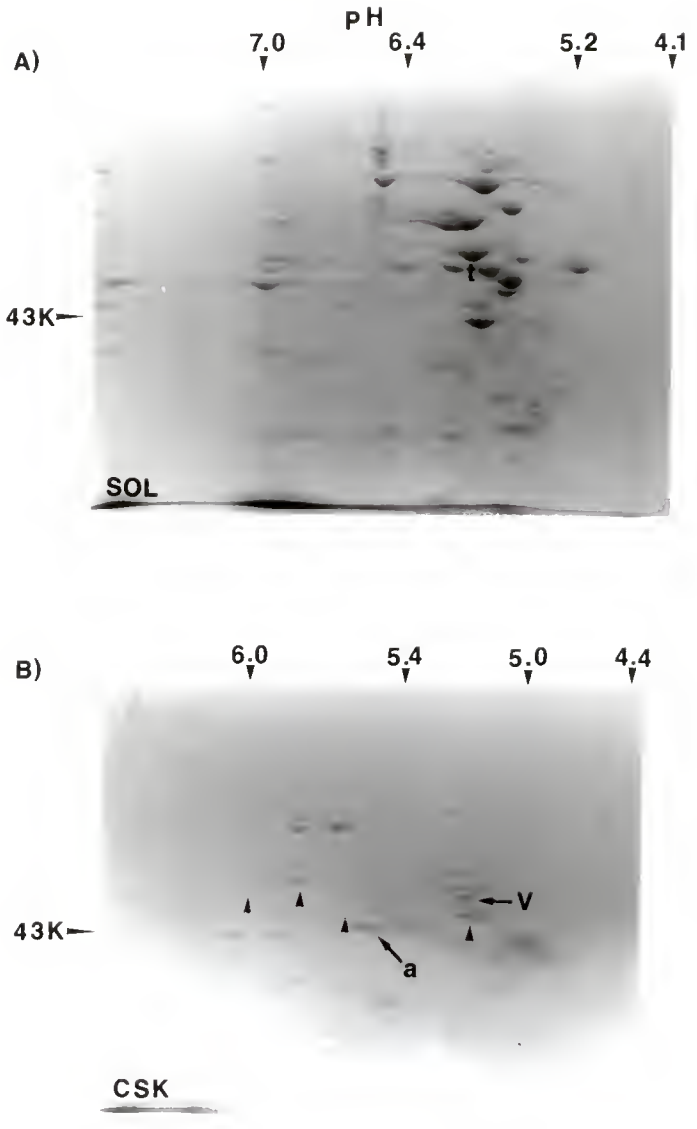
in Figure 3-2, the cytoskeleton fraction clearly contained actin as well as the major HeLa cytokeratins (Franke et al., 1981). In addition, vimentin appeared to be enriched in the cytoskeleton, which was confirmed by immunoblot analysis. Extraction of the cytoskeleton required a cold, calcium containing buffer and consequently the tubulins were solubilized and therefore absent in the Csk fraction. The tubulin proteins were clearly contained in the soluble fraction as determined by immunoblot analysis. Additional support for separation of cytoskeleton and soluble phases comes from the observation that the bulk of the tRNA species is in the soluble phase which is in agreement with previously reported values (data not shown; Cervera et al. 1983). Although the HLA-B7 messenger RNA is found on membrane-bound polysomes and the histone mRNA on nonmembrane-bound polysomes, both species of messenger RNAs are associated almost exclusively with the cytoskeletal fraction. Nearly 97% of the HLA-B7 mRNA and approximately 85-90% of the H3 and H4 histone messages are retained in the Csk (Fig. 3-1; Table 3-1).

The Effects of Metabolic Inhibitors on the Subcellular Localization of Histone and HLA-B7 mRNA

Detectable levels of histone mRNA were observed in each of the subcellular samples, including the Sol fraction and the membrane-bound polysomal fractions. However, the HLA-B7

Figure 3-2. Two-dimensional gel electrophoresis of cytoskeleton and soluble associated proteins.

HeLa cells were fractionated into a cytoskeleton and soluble phase, protein was extracted from each phase and subsequently analyzed by two-dimensional gel electrophoresis as described in Chapter 2. Proteins of the soluble fraction (Sol), A, were separated using wide range (pH 3-7) ampholines while cytoskeletal proteins (Csk), B, were separated with narrow range (pH 5-7) ampholines to more clearly identify cytoskeletal species. V: vimentin, a: actin, and t: tubulins. Unlabeled arrows in B depict HeLa cytokeratins of 54 Kd (pI 6.0); 52.5 Kd (pI 6.1); 46 Kd (pI 5.1); and 45 Kd (pI 5.7), which were spots highly reactive with commercially prepared prekeratin or stratum corneum antisera (Franke et al., 1981).


pH
A)
7.0
6.4
5.2
4.1
43K
t
SOL
B)
6.0
5.4
5.0
4.4
43K
V
a
CSK

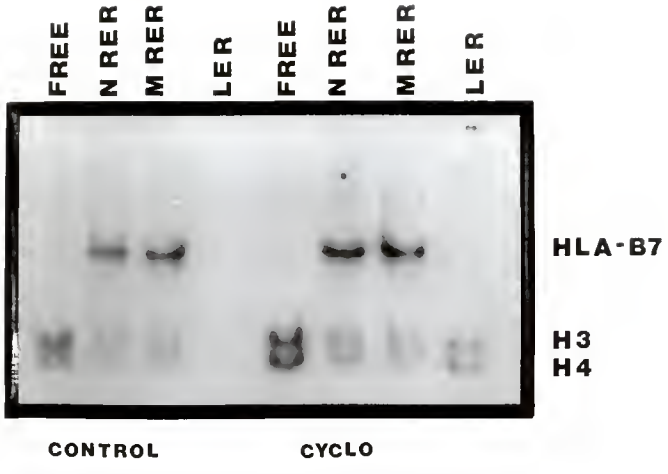
mRNA was located almost exclusively in the cytoskeleton and membrane-bound polysome fractions. The widespread subcellular localization of histone mRNA may be due to non-specific trapping of nonmembrane-bound polysomes in each of the fractions during the isolation procedure. Alternatively, this observation may reflect the presence of mRNAs encoding variant histone proteins that could be targeted to the different cytoplasmic compartments. Inhibition of DNA synthesis with hydroxyurea results in a rapid destabilization of cell cycle dependent histone mRNAs, whereas cell cycle independent histone mRNAs remain stable in the presence of this drug (Baumbach et al., 1984; Heintz et al., 1983; DeLisle et al., 1983; Graves and Marzluff, 1984; Plumb et al., 1983). Conversely, inhibition of protein synthesis with cycloheximide results in a 2-3 fold increase in cytoplasmic levels of histone mRNAs (Butler and Mueller, 1973; Baumbach et al., 1984). Differential sensitivity of histone mRNAs in the subcellular fractions to hydroxyurea or cycloheximide treatment would favor the latter suggestion that cell cycle dependent and independent histone gene transcripts may selectively reside in specific cytoplasmic compartments.

Treatment of exponentially growing HeLa cells with hydroxyurea, as described in Chapter 2, resulted in a complete and selective destabilization of histone mRNA in the LER, nonmembrane-bound and membrane-bound polysomal

fractions (Figure 3-3). This is in sharp contrast to the HLA-B7 mRNA which appeared to be fully stable under these conditions. Cycloheximide treatment had a stabilizing effect on the levels of histone mRNA in each subcellular fraction (Figure 3-3). When compared with control HeLa cells, cyclohéximide treatment resulted in a 2.1 fold (H4) and a 2.4 fold (H3) increase in the levels of histone mRNA in the nonmembrane-bound polysomal fraction. Similar increases were also seen for levels of histone mRNAs in the LER and membrane-bound polysomal fractions. Inhibition of protein synthesis with cycloheximide followed by DNA synthesis inhibition with hydroxyurea resulted in the stabilization of histone mRNA in each subcellular fraction, whereas the stability of HLA-B7 mRNA remained unaffected (Figure 3-3). Similar results were obtained when these inhibitor studies were expanded to include the cytoskeleton and soluble fractions (Figure 3-4). Cycloheximide treatment resulted in a 2-3 fold increase in the representation of H3 and H4 histone mRNA over untreated cultures in both the cytoskeleton and soluble fractions. HLA-B7 mRNA appeared to be only moderately stabilized in the presence of this drug. When cells were treated with hydroxyurea, histone mRNA levels were greatly reduced in both the cytoskeleton and soluble fractions whereas the HLA-B7 mRNA was found in comparable levels to those present in the untreated samples. Consistent with previous data, cotreatment of HeLa cells

Figure 3-3. Northern blot analysis of H3 histone, H4 histone and HLA-B7 mRNAs isolated from subcellular fractions of cells treated with metabolic inhibitors.

Exponentially growing HeLa cells were incubated in the presence of metabolic inhibitors and fractionated into nonmembrane-bound and membrane-bound polysomes as described in Chapter 2. RNA was extracted from each fraction and assayed for H3 histone mRNA, H4 histone mRNA and HLA-B7 mRNA content by Northern blot analysis as described in Figure 3-1 and Chapter 2. Upper panel from left to right, lanes: 1) control (untreated), nonmembrane-bound polysomes; 2) control, NRER; 3) control, MRER; 4) control, LER (omitted); 5) cycloheximide (Cy), nonmembrane-bound polysomes; 6) Cy, NRER; 7) Cy, MRER; and 8) Cy, LER. Lower panel from left to right, lanes: 1) hydroxyurea (HU), nonmembrane-bound polysomes; 2) Hu, NRER; 3) Hu, MRER; 4) Hu, LER; 5) CY/HU, nonmembrane-bound polysomes; 6) Cy/Hu, NRER; 7) Cy/Hu, MRER; and 8) Cy/Hu, LER.


FREE
N RER
M RER
LER
FREE
N RER
M RER
LER
HLA-B7
H3
H4
CONTROL
CYCLO


FREE
N RER
MRER
LER
FREE
NRER
M RER
LER
HLA-B7
H3
H4
HYDROXY
CYC/HYDROXY

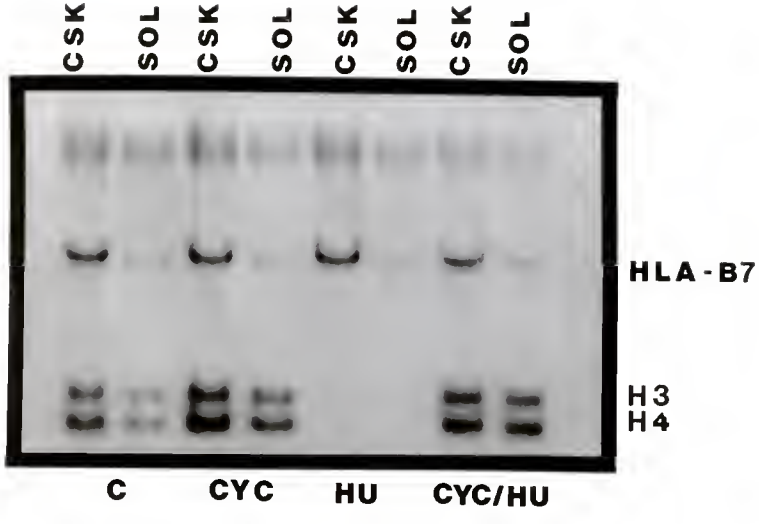
with cycloheximide and hydroxyurea had a stabilizing effect on histone mRNA levels and no apparent effect on HLA-B7 mRNA stability in both subcellular fractions. There were no obvious differences in the stability of histone mRNAs localized in the various subcellular compartments when treated with hydroxyurea or cycloheximide. It is therefore reasonable to conclude that the histone mRNA isolated in the LER and membrane-bound fractions is a consequence of the subcellular fractionation procedure. Our results, taken together with previous findings (Jacobs-Lorena et al., 1972; Liautard and Jeanteur, 1979; Gallwitz and Breindl, 1972; Borun et al., 1975; Stein et al., 1975) suggest that histone mRNAs, during normal cellular processing, are targeted to the nonmembrane-bound polysomes, the common site of histone protein synthesis.

DISCUSSION

Our objectives in pursuing these studies were two-fold. The first was to confirm that histone proteins are synthesized on nonmembrane-bound polysomes. The second was to determine if the histone mRNA-containing polysomes are associated with the cytoskeleton. Our rationale for this approach was to begin addressing the questions whether the subcellular location of histone containing polysomes contributes to: a) the ability of histone mRNAs to be translated immediately following transcription, b) the rapid

Figure 3-4. Northern blot analysis of H3 histone, H4 histone and HLA-B7 mRNAs associated with the cytoskeleton and soluble fractions from HeLa cell treated with metabolic inhibitors.

Exponentially growing HeLa cells were incubated in the presence of metabolic inhibitors and fractionated into cytoskeleton and soluble fractions as described in Chapter 2. RNA was extracted and assayed for H3 histone, H4 histone and HLA-B7 mRNA content by Northern blot analysis as described in Figure 3-1. From left to right, lanes: 1) control, Csk; 2) control, Sol; 3) cycloheximide (Cy), Csk; 4) Cy, Sol; 5) hydroxyurea (HU), Csk; 6) HU, Sol; 7) Cy/HU, Csk; and 8) Cy/HU, Sol.


CSK
SOL
CSK
SOL
CSK
SOL
CSK
SOL
HLA-B7
H3
H4
C
CYC
HU
CYC/HU

shuttling of newly synthesized histone polypeptides into the nucleus and c) histone mRNA stability.

In agreement with long-standing in vitro translation and cDNA hybridization results (Jacobs-Lorena et al., 1972; Liautard and Jeanteur, 1979; Gallwitz and Briendl, 1972; Borun et al., 1975; Stein et al., 1975), we find that histone mRNAs reside predominantly on nonmembrane-bound polysomes. While we cannot dismiss the possibility that the amounts of histone mRNAs detected in membrane-bound polysomal subcellular fractions are biologically significant (see Figures 3-1 and 3-3), we feel that this is a consequence of the isolation procedure which operationally defines the fraction obtained. The absence of detectable HLA-B7 mRNAs on nonmembrane-bound polysomes suggests that while mRNAs encoding intracellular proteins may be trapped by the endoplasmic reticulum during this subcellular fractionation technique, there is little, if any, stripping of membrane-bound polysomes encoding extracellular polypeptides, such as the HLA-B7 class I antigen. Within this context, the question arises whether the transcripts from histone genes that are not expressed in a cell cycle-dependent manner (approximately 8-10%) are represented on membrane-bound polysomes. However, this is not a likely possibility because inhibition of DNA replication with hydroxyurea brings about a comparable degradation of histone mRNAs in all the subcellular fractions examined.

The association of histone mRNAs with the cytoskeleton (Figure 3-1) is consistent with studies carried out by Penman and co-workers in which they examined the subcellular localization of vesicular stomatitis virus (VSV) mRNAs in HeLa cells following viral infection (Cervera et al., 1981). These investigators observed that in VSV-infected HeLa cells both the viral mRNA coding for the G viral glycoprotein and the nonmembrane-bound polysomal viral mRNAs were associated with the cytoskeleton. While the specific nature of the association of polysomes with the cytoskeleton remains to be definitively established, the ability to release ribosomes and retain mRNAs on the cytoskeleton suggests that there may be a direct attachment of mRNAs to the cytoskeleton (Lenk et al., 1977; Lenk and Penman, 1979; Lemieux and Beaud, 1982). It is therefore reasonable to speculate that the association of mRNAs with the cytoskeleton plays a functional role in protein synthesis and/or in the regulation of mRNA stability. This is a particularly attractive possibility because the association of histone mRNAs with the cytoskeleton could permit the compartmentalization of histone mRNA-containing polysomes in a particular region of the cytoplasm. The potential for compartmentalization afforded by the cytoskeleton may also be related to the rapid and selective destabilization of histone mRNAs at the natural end of S phase or following inhibition of DNA synthesis.

As with the protocol utilized to separate nonmembrane-bound and membrane-bound polysomes, the cytoskeleton preparation obtained must be operationally defined. While this does not necessarily detract from the biological relevance, differences in cytoskeleton preparations are undoubtedly reflected in the biochemical composition of the isolated complex. Additional characterization of the cytoskeleton preparation with respect to possible variations in regions of the complex may provide insight into mechanisms by which specific mRNAs may be localized and their functional properties selectively modulated.

CHAPTER 4

SUBCELLULAR LOCATION OF HISTONE mRNA PLAYS A ROLE IN THE POSTTRANSCRIPTIONAL REGULATION OF HISTONE GENE EXPRESSION

Introduction

The association of histone mRNA-containing nonmembrane-bound polysomes with the cytoskeleton may provide a structural basis for the localization of the mRNA in specific regions of the cytoplasm. Consistent with this reasoning, the factors that mediate the rapid and selective destabilization of histone mRNA during inhibition of DNA synthesis may be co-localized with the message in the same subcellular compartment. Therefore, the subcellular localization of histone mRNA may play an important role in its posttranscriptional regulation.

In this chapter, we describe a biological approach, using a series of signal peptide-histone fusion genes, to study whether the subcellular location of histone mRNA-containing polysomes is functionally related to the coupling of histone mRNA stability with DNA replication. In order to study the influence of subcellular location on histone mRNA stability, we constructed a signal peptide-histone gene which was designed to target the encoded fusion message to

membrane-bound polysomes. The ability of the cell to selectively recognize and destabilize histone mRNA in a foreign subcellular compartment could then be examined. Based on the data presented here, we propose that the subcellular location of histone mRNA, with respect to the class of polysomes in which they are associated with, plays a significant role in the coupling of histone mRNA stability with DNA replication.

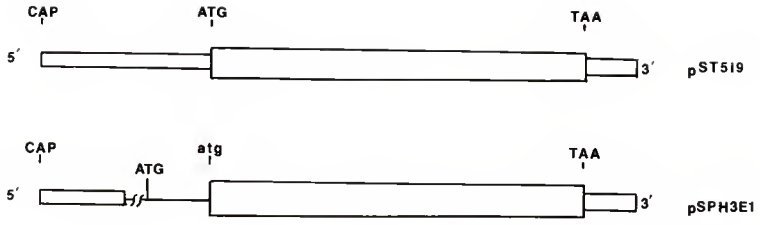
Results

The construction of the signal peptide-histone fusion gene is described in Chapter 2 and outlined in Figure 2-1. The upstream flanking region of the chimeric gene contains 210 base pairs of the 5' regulatory sequences of the cell cycle-dependent human H3 histone gene pST519 including the TATAA and CCAAT consensus sequences. This region is followed by the H3 mRNA cap site and sequences encoding the initial 20 nucleotides of the non-translated H3 histone leader. The fusion gene is, therefore, under transcriptional control of the H3 histone gene promoter, and a segment of the H3 leader which has been implicated in the coupling of histone mRNA levels to DNA synthesis is present (Morris et al., 1986). The H3 histone leader segment is fused to the untranslated leader sequences of the beta-lactamase signal peptide including the ATG translation start codon. The H3 histone structural gene is fused in frame to the signal peptide

coding sequences and extends approximately 300 base pairs beyond the TAA translation stop codon, including a region which has been implicated in histone mRNA destabilization when DNA synthesis is inhibited (Luscher et al., 1985; Pandey and Marzluff, 1987; Graves et al., 1987). An SV40 enhancer element was incorporated into the upstream EcoRI site in the recombinant plasmid to increase cellular levels of the fusion transcript. The mRNA encoded by the fusion gene consists of the first 20 nucleotides of the H3 histone 5' leader sequences followed by the untranslated leader sequence of the beta-lactamase signal peptide, the entire beta-lactamase signal peptide coding sequences (including the translation start codon), the entire H3 histone coding region which is fused in frame to the signal peptide coding region, and the H3 histone 3' untranslated region (Figure 4-1). The junction between the signal peptide coding region and the H3 histone coding region has been sequenced by Sanger's dideoxy method and the reading frame has been conserved. All sequences required for the synthesis and processing of the chimeric mRNA, as well as for translation of the fusion protein, are present. The signal peptide-H3 histone fusion gene with a single SV40 enhancer element is designated pSPH3E1 and an identical fusion construct containing multiple SV40 enhancer elements in the EcoRI site is designated pSPH3E2.

Figure 4-1. Schematic diagram of endogenous H3 histone mRNA ST519 and the beta-lactamase signal peptide-H3 histone fusion mRNA.

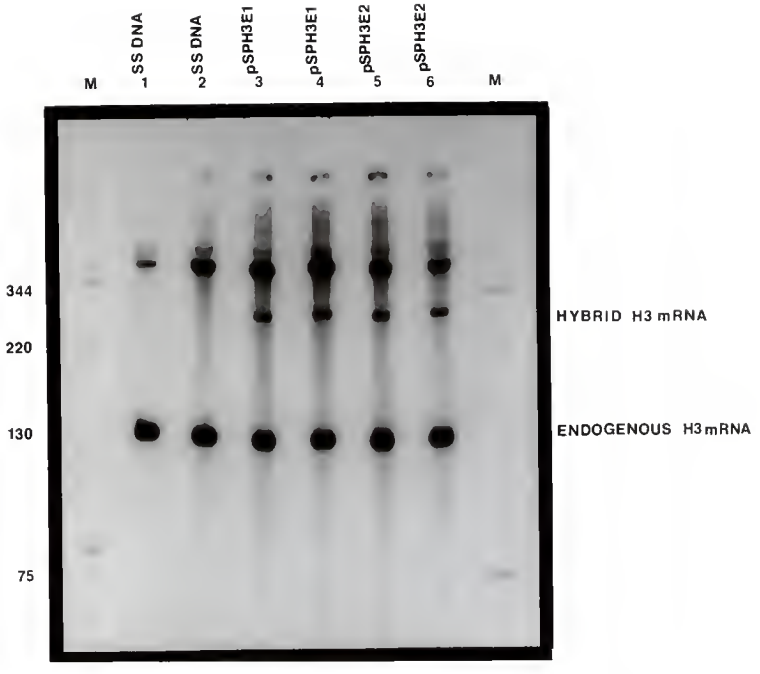
The signal peptide-histone fusion mRNA is identical to the wild type histone mRNA with the exception of the signal peptide encoded sequences that have been inserted in frame into the histone gene. The 5' mRNA sequences and the 3' mRNA sequences that have been implicated in coupling histone mRNA stability to DNA synthesis are retained in place in the signal peptide-histone fusion mRNA (Morris et al., 1986; Pandey and Marzluff, 1987). Boxed areas represent histone H3 mRNA sequences, single lined areas represent beta-lactamase signal peptide derived sequences.


CAP
ATG
TAA
5′
3′
pST5I9
CAP
atg
ATG
TAA
5′
3′
pSPH3E1

To test for expression of the signal peptide-histone fusion gene in human cells, we transfected pSPH3E1 and pSPH3E2 DNA into HeLa cell monolayers by the calcium phosphate precipitation method as described in Chapter 2 (Gorman et al., 1982; Graham and van der Eb, 1973). Forty-six hours post-transfection cells were harvested and total cellular RNA was isolated. The RNA was subjected to S1 nuclease protection analysis by a modification of Berk and Sharp (1978). The probe used in the S1 nuclease assays was the SmaI fragment from pSPH3E1 which was radiolabeled at its 5' termini as described in Chapter 2. The probe is complimentary to endogenous HeLa histone H3 mRNA from the 5' end-labeled SmaI site within the protein coding region to the signal peptide-histone fusion junction and therefore protects a 130 nucleotide region of the H3 mRNA from S1 nuclease digestion. As seen in Figure 4-2, when total cellular RNA from two independent transfections of HeLa cells with pSPH3E1 or pSPH3E2 DNA was analyzed by S1 nuclease digestion, a 130 nucleotide fragment corresponding to endogenous H3 histone mRNA was protected as well as an approximately 280 nucleotide fragment corresponding to the signal peptide-histone fusion mRNA species (lanes 3-6). In HeLa cells transfected with salmon sperm DNA, only the 130 nucleotide fragment was detected (lanes 1 and 2). These data demonstrate that the signal peptide-histone fusion gene is capable of expression in HeLa cells and that the mRNA

Figure 4-2. Expression of the signal peptide-histone fusion gene in HeLa cells.

The expression of the signal peptide-histone fusion gene was tested in a short term transient assay and analyzed by an S1 nuclease protection assay. HeLa cell monolayers were transfected with 20 μg DNA according to Gorman et al. (1982), in a calcium phosphate/DNA complex prepared as described by Graham and van der Eb (1973). The transfected cells were incubated at 37°C, 5% CO_2 for 46 hours following transfection. The cells were then harvested, total cellular RNA was isolated and analyzed by S1 nuclease protection assay (200 μg RNA per sample) as described in Chapter 2. Lanes 1 and 2, HeLa cells transfected with salmon sperm DNA; lanes 3 and 4, HeLa cells transfected with pSPH3E1 DNA; and lanes 5 and 6, HeLa cells transfected with pSPH3E2 DNA. Marker lanes (M) are radiolabeled HinfI digests of pBR322 DNA. Each lane represents RNA isolated from an independently transfected cell culture.

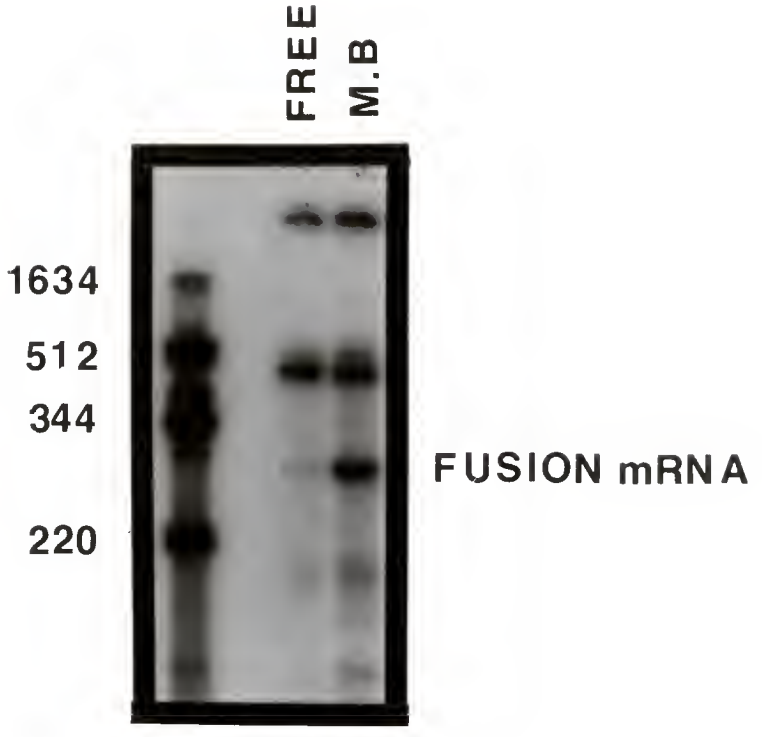

M
SS DNA 1
SS DNA 2
pSPH3E1 3
pSPH3E1 4
pSPH3E2 5
pSPH3E2 6
M
344
220
130
75
HYBRID H3 mRNA
ENDOGENOUS H3 mRNA

transcribed from this gene is sufficiently stable to be detected in a short-term transient transfection assay.

To determine the class of polysomes with which the signal peptide-histone fusion mRNAs are associated, HeLa cells were transfected with pSPH3E1 and 46 hours later nonmembrane-bound and membrane-bound polysomes were isolated as described in Chapter 2. RNA was isolated from both classes of polysomes and analyzed by S1 nuclease protection assay using the SmaI fragment of the signal peptide-histone fusion gene (^{32}P-5'-labeled). As seen in Figure 4-3, approximately 30% of the signal peptide-histone fusion mRNA was found in the nonmembrane-bound polysome fraction and approximately 70% was associated with the membrane-bound polysomes (lanes 1 and 2 respectively). This is in contrast to endogenous H3 histone mRNA which is represented by approximately 90% in the nonmembrane-bound polysome and 10% in the membrane-bound polysome fraction. The percent distribution of the histone chimeric mRNA within the polysomal RNA fractions was determined by densitometric analysis of the autoradiograms and the values corrected for the total yield of RNA in each subcellular fraction. While the extent to which the signal peptide-histone fusion mRNA is represented on the membrane-bound polysomes varied from experiment to experiment, we always observed 60-90% of the fusion mRNA in the membrane-bound polysome fraction (n=6).

Figure 4-3. The subcellular localization of the signal peptide-histone fusion mRNA.

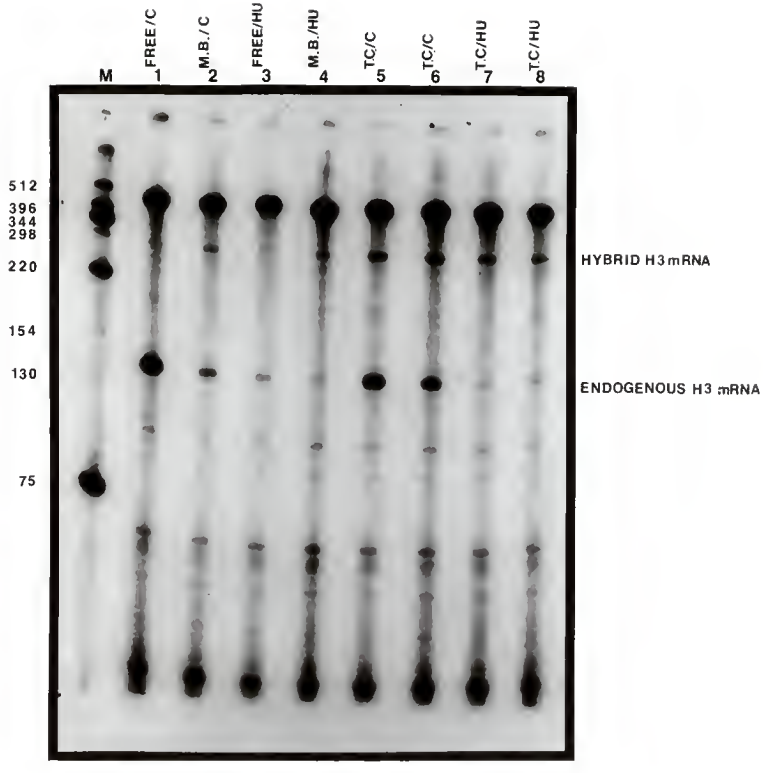
HeLa cell monolayers were transfected with pSPH3E1 and cultured as described in Chapter 2. Forty-six hours after transfection, cell cultures were harvested and nonmembrane-bound and membrane-bound polysomes were isolated. The RNAs from these fractions were analyzed by the S1 nuclease protection assay (5 μg RNA per sample). The distribution of mRNA species within the subcellular fractions was quantitated by scanning laser densitometric analysis of the autoradiograms. Lanes: 1) pBR322 HinfI molecular weight marker; 2) nonmembrane-bound polysomal RNA; and 3) membrane-bound polysomal RNA.


FREE
M.B
1634
512
344
220
FUSION mRNA

The ability to direct a cell cycle-dependent histone mRNA to membrane-bound polysomes provided the possibility to address the involvement of subcellular location in the coupling of histone mRNA stability with DNA replication. As seen in Figure 4-4 (lanes 1-4), endogenous histone mRNA levels were reduced by greater than 90%, as determined by densitometric analysis of the autoradiogram, in HeLa cells following inhibition of DNA replication by treatment with 1 mM hydroxyurea for 60 minutes. In contrast, inhibition of DNA replication by hydroxyurea treatment does not result in a reduction of fusion message levels on membrane-bound polysomes (lane 4). The finding that the signal peptide-histone fusion mRNA is stable following DNA synthesis inhibition is further supported by the S1 nuclease analysis of total cellular RNA (Figure 4-4). The endogenous histone mRNA levels in hydroxyurea treated HeLa cells were reduced by approximately 95% compared with those in untreated HeLa cells (lanes 5-8) (with each lane representing an independent transfected cell culture). In contrast, only a 4% reduction in fusion mRNA levels was measured (lanes 5-8). These results demonstrate that the incorporation of the E. coli beta-lactamase signal peptide into a human H3 histone gene is sufficient to target the encoded fusion mRNA to the membrane-bound polysomes and to confer stability to the fusion message when DNA synthesis is inhibited.

Figure 4-4. The stability of the signal peptide-histone fusion mRNA following inhibition of DNA synthesis.

HeLa cell monolayers were transfected with pSPH3E1 DNA and cultured as described in Chapter 2. Forty-six hours after transfection, half of the transfected HeLa cell cultures were treated with 1 mM hydroxyurea in completed medium for 1 hour at 37°C, 5% CO_2. Cell cultures were harvested and nonmembrane-bound and membrane-bound polysomes were isolated. Total cellular RNA from both the control and hydroxyurea-treated samples was also isolated. The RNA was analyzed by the S1 nuclease protection assay as described in Chapter 2, using 5 μg RNA per sample. The distribution of mRNA species within the subcellular fractions was quantitated by densitometric analysis of the autoradiograms. Lanes: 1) control (untreated cells), nonmembrane-bound polysomes; 2) control, membrane-bound polysomes; 3) hydroxyurea, nonmembrane-bound polysomes; 4) hydroxyurea, membrane-bound polysomes; 5) and 6) control, total cellular RNA; and 7) and 8) hydroxyurea, total cellular RNA. Lanes 5-8 represent RNA isolated from independently transfected cell cultures.

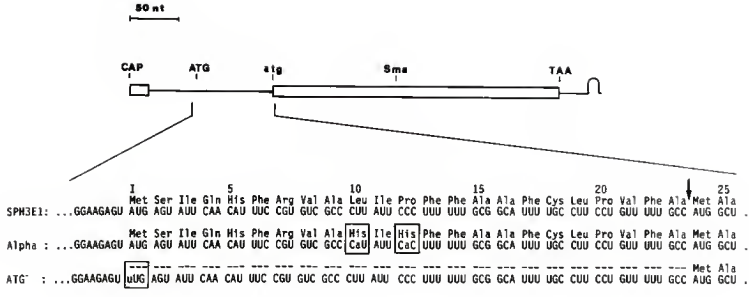

M
FREE/C
1
M.B./C
2
FREE/HU
3
M.B./HU
4
TC/C
5
TC/C
6
TC/HU
7
TC/HU
8
512
396
344
298
220
154
130
75
HYBRID H3 mRNA
ENDOGENOUS H3 mRNA

It was possible that the stability of the histone fusion mRNA during inhibition of DNA synthesis was due to the targeting of the mRNA to a foreign subcellular compartment, namely the membrane-bound polysomes. Alternatively, the nucleotide sequences coding for the signal peptide may have disrupted the histone mRNA structure in such a way that it was no longer recognized by the factors involved in the destabilization of histone mRNA. To test these possibilities, the signal peptide was mutated in order to retain the fusion mRNA, without significantly changing the mRNA structure, on nonmembrane-bound polysomes. If the class of polysomes plays a role in the coupling of histone mRNA stability with DNA replication then the mutated fusion mRNAs that are retained on nonmembrane-bound polysomes should be efficiently destabilized during inhibition of DNA synthesis. Conversely, if the mRNA structure has been disrupted by the nucleotide sequences encoding the signal peptide then the mutated histone fusion mRNAs localized on nonmembrane-bound polysomes should be stable during inhibition DNA synthesis.

The signal peptide-histone fusion gene was mutated by site directed mutagenesis (Zoller and Smith, 1983) as described in Chapter 2 and the corresponding mRNAs are schematically diagrammed in Figure 4-5. The first mutant, pSPH3E1alpha, contains two point mutations in the region coding for the hydrophobic domain of the signal peptide,

Figure 4-5. Schematic diagram of wild type and mutated signal peptide-histone fusion mRNAs.

The signal peptide-histone fusion gene (SPH3E1) was mutated by site directed mutagenesis (Zoller and Smith, 1983) as described in Chapter 2. Abbreviations: H3, endogenous H3 histone mRNA; SPH3E1, signal peptide-histone fusion mRNA; SPH3E1alpha, signal peptide-histone fusion mRNA mutated in the hydrophobic region of the signal peptide (leu at position 10 and pro at position 12 substituted with his); SPH3E1ATG$^-$, signal peptide-histone fusion mRNA mutated in the signal peptide translation start codon; CAP, 5' cap structure; ATG, predicted translation start codon; atg, internal translation start codon for histone H3 protein; TAA, translation stop codon.


50 nt
CAP
ATG
atg
Sma
TAA
I
5
10
15
20
25
Met Ser Ile Gln His Phe Arg Val Ala Leu Ile Pro Phe Phe Ala Ala Phe Cys Leu Pro Val Phe Ala Met Ala
SPH3E1: ...GGAAGAGU AUG AGU AUU CAA CAU UUC CGU GUC GCC CUU AUU CCC UUU UUU GCG GCA UUU UGC CUU CCU GUU UUU GCC AUG GCU ...
Met Ser Ile Gln His Phe Arg Val Ala His Ile His Phe Phe Ala Ala Phe Cys Leu Pro Val Phe Ala Met Ala
Alpha : ...GGAAGAGU AUG AGU AUU CAA CAU UUC CGU GUC GCC CaU AUU CaC UUU UUU GCG GCA UUU UGC CUU CCU GUU UUU GCC AUG GCU ...
--- Met Ala
ATG⁻ : ...GGAAGAGU uUG AGU AUU CAA CAU UUC CGU GUC GCC CUU AUU CCC UUU UUU GCG GCA UUU UGC CUU CCU GUU UUU GCC AUG GCU ...

which results in the substitution of leucine at amino acid position 10 (GAA→GTA) and proline at position 12 (GGG→GTG) with positively charged histidine residues. The second mutant, pSPH3E1ATG$^-$, contains a single nucleotide substitution which destroys the ATG translation initiation codon of the signal peptide (ATG→TTG). This mutation should result in the initiation of translation at the natural ATG codon of the histone coding region without the synthesis of the signal peptide.

The distribution of the SPH3E1, SPH3E1alpha and SPH3E1ATG$^-$ signal peptide-histone fusion mRNAs in nonmembrane-bound and membrane-bound polysome fractions from transfected HeLa monolayer cell cultures was determined by S1 nuclease protection analysis. As seen in Figure 4-6, the SPH3E1ATG$^-$ mRNA partitioned into the nonmembrane-bound polysome fraction to the same extent as endogenous histone H3 mRNA. Approximately 82% of the SPH3E1ATG$^-$ mRNA and 83% endogenous histone mRNA was found associated with the nonmembrane-bound polysomes (Table 4-1). In contrast, SPH3E1 mRNA was predominantly associated with membrane-bound polysomes (Fig. 4-6). Approximately 68% of SPH3E1 mRNA was localized in the membrane-bound polysomal RNA fraction (Table 4-1). The SPH3E1alpha mRNA displayed a more intermediate association with membrane-bound polysomes; approximately 40% of the SPH3E1alpha mRNA was localized in the membrane-bound polysome fraction and 60% in the nonmembrane-bound polysomal fraction (Fig. 4-6 and Table 4-

Figure 4-6. Distribution of SPH3E1, SPH3E1alpha, and SPH3E1ATG$^-$ mRNAs in nonmembrane-bound and membrane-bound polysomal fractions.

HeLa cell monolayers were transfected with the signal peptide-histone fusion genes by the calcium phosphate precipitation method and 46 hours posttransfection nonmembrane-bound and membrane-bound polysomes were isolated. RNA (5 μg) from each subcellular fraction was assayed for signal peptide-histone fusion mRNA and endogenous H3 histone mRNA content by S1 nuclease protection analysis as described in Chapter 2. Abbreviations: SPH3E1, wild type signal peptide-histone fusion mRNA; alpha, hydrophobic mutant of SPH3E1; ATG, signal peptide translation initiation codon mutant of SPH3E1. Lane 1, nonmembrane-bound polysomal RNA; Lane 2, membrane-bound polysomal RNA.

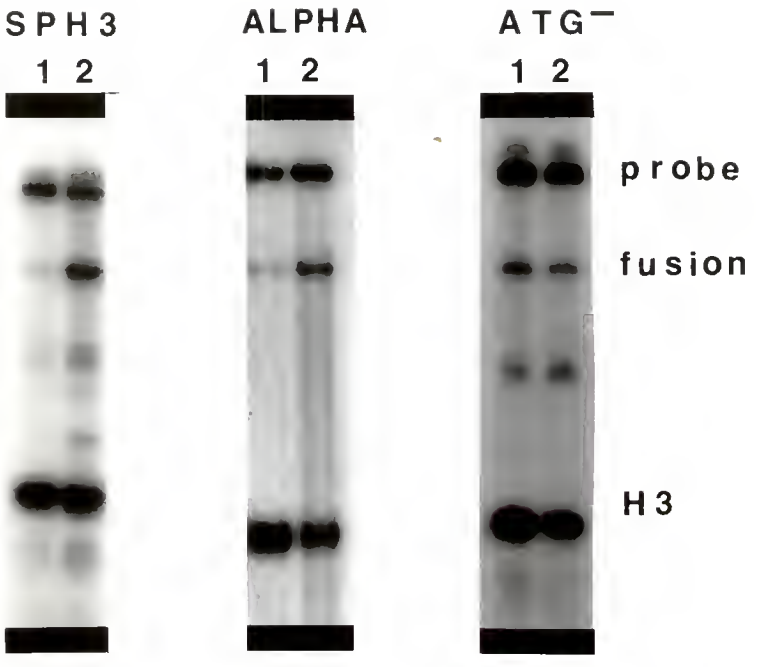

SPH3
1 2
ALPHA
1 2
ATG⁻
1 2
probe
fusion
H3

Table 4-1. Quantitation of SPH3E1, SPH3E1alpha and SPH3E1ATG$^-$ mRNA in nonmembrane-bound and membrane-bound polysome fractions.

	Nonmembrane-bound	Membrane-bound
SPH3E1 mRNA	32%	68%
SPH3E1alpha mRNA	60%	40%
SPH3E1ATG$^-$ mRNA	82%	18%
Endogenous H3 mRNA	83%	17%

The distribution of SPH3E1, SPH3E1alpha, SPH3E1ATG$^-$, and endogenous H3 histone mRNAs in nonmembrane-bound and membrane-bound polysome fractions was quantitated by densitometric analysis of the S1 nuclease protection assay presented in Figure 4-6. The densitometric values obtained represent the quantity of mRNA in 5 μg RNA. The values were then adjusted to reflect the total yield of RNA recovered in each fraction.

1). These results indicate that the mutation of the ATG translation start codon of the signal peptide results in the synthesis of a signal peptide-histone fusion mRNA (SPH3E1ATG$^-$) that is associated with nonmembrane-bound polysomes. In addition, the incorporation of two histidine amino acid residues in the hydrophobic domain of the signal peptide partially blocks the translocation of the mRNA from nonmembrane-bound polysomes to membrane-bound polysomes.

To study the stability of the mutated signal peptide-histone fusion mRNAs during inhibition of DNA synthesis, the genes (SPH3E1, SPH3E1alpha, and SPH3E1ATG$^-$) were transfected into HeLa cell monolayers by the calcium phosphate precipitation method as described in Chapter 2. Forty-six hours posttransfection, the cells were treated with 1 mM hydroxyurea and samples were taken at 20 minute time intervals. Total cellular RNA was isolated and assayed for signal peptide-histone fusion mRNA and endogenous H3 histone mRNA content by S1 nuclease protection analysis as described in Chapter 2. As seen in Figure 4-7 and summarized in Figure 4-10, inhibition of DNA synthesis for one hour resulted in the destabilization of only 46% of SPH3E1 mRNA as compared to the control cell culture (lanes 1,2 and 7,8). In sharp contrast, SPH3E1ATG$^-$ mRNA was destabilized by 94%, which is to the same extent as measured for endogenous H3 histone mRNA (Figure 4-8 lanes 1,2 and 7,8; Fig. 4-10). Inhibition of DNA synthesis for one hour with 1 mM hydroxyurea

Figure 4-7. Effects of hydroxyurea treatment on SPH3E1 mRNA.

HeLa cell monolayers were transfected with pSPH3E1 DNA and 46 hours post-transfection were treated with 1 mM hydroxyurea (HU). Cells were harvested at 20 minute intervals and total cellular RNA was prepared. The RNA (10 μg) was subjected to S1 nuclease protection analysis to quantitate the levels of wild type signal peptide-histone fusion mRNA and endogenous H3 histone mRNA. Each lane represents an individually transfected cell culture. MW is radiolabeled Hpa II digest of pBR322 DNA. Lanes 1 and 2, control; lanes 3 and 4, 1 mM HU 20 minutes; lanes 5 and 6, 1 mM HU 40 minutes; lanes 7 and 8, 1 mM HU 60 minutes.

SPH3E1

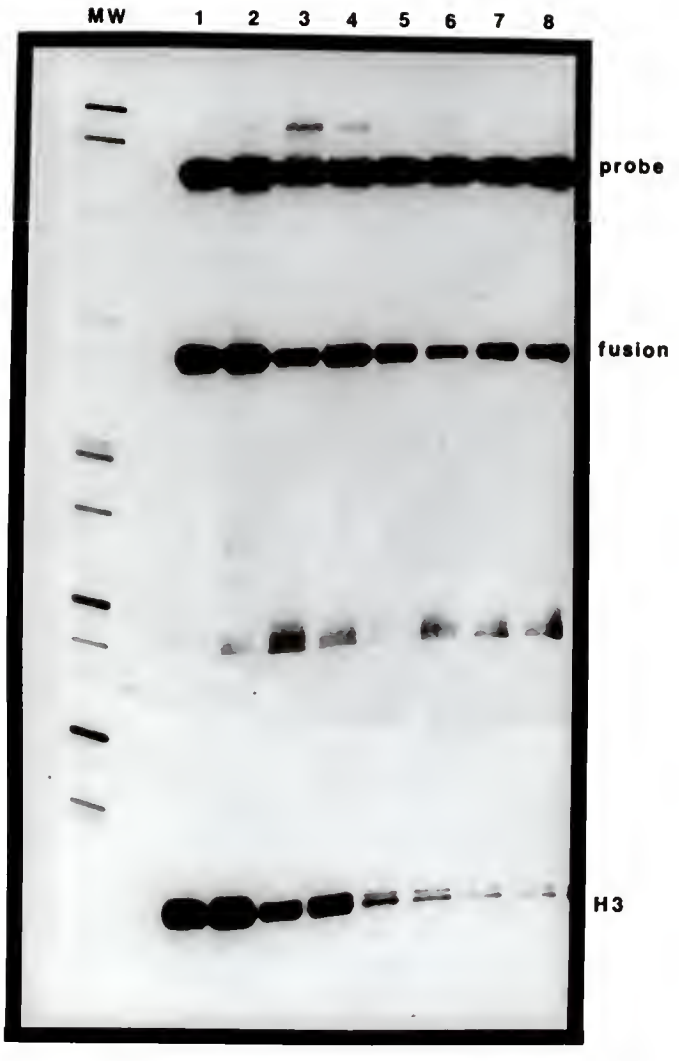


Figure 4-8. Effects of hydroxyurea treatment on SPH3E1ATG$^-$ mRNA.

HeLa cell monolayers were transfected with pSPH3E1ATG$^-$ DNA and 46 hours post-transfection were treated with 1 mM hydroxyurea (HU). Cells were harvested at 20 minute intervals and total cellular RNA was prepared. The RNA (10 μg) was subjected to S1 nuclease protection analysis to quantitate the levels of SPH3E1ATG$^-$ mRNA and endogenous H3 histone mRNA. Each lane represents an individually transfected cell culture. MW is radiolabeled Hpa II digest of pBR322 DNA. Lanes 1 and 2, control; lanes 3 and 4, 1 mM HU 20 minutes; lanes 5 and 6, 1 mM HU 40 minutes; lanes 7 and 8, 1 mM HU 60 minutes.

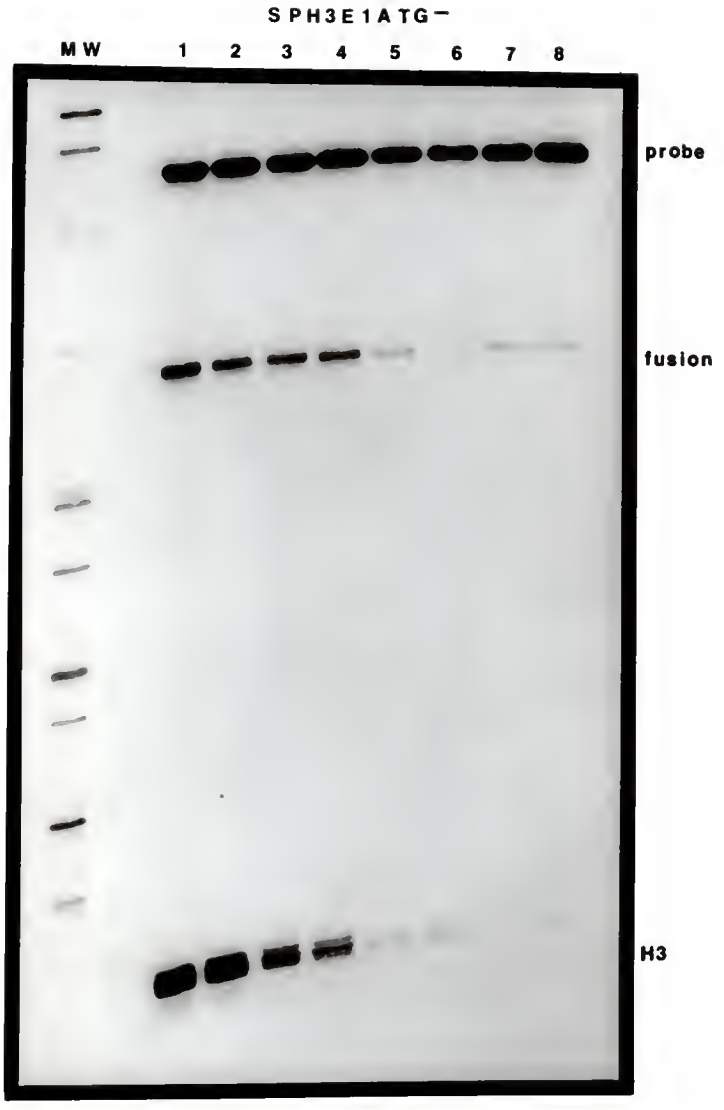

SPH3E1ATG−
MW
1
2
3
4
5
6
7
8
probe
fusion
H3

Figure 4-9. Effects of hydroxyurea treatment on SPH3E1alpha mRNA.

HeLa cell monolayers were transfected with pSPH3E1alpha DNA and 46 hours post-transfection were treated with 1 mM hydroxyurea (HU). Cells were harvested at 20 minute intervals and total cellular RNA was prepared. The RNA (10 μg) was subjected to S1 nuclease protection analysis to quantitate the levels of the SPH3E1alpha mRNA and endogenous H3 histone fusion mRNA. Each lane represents an individually transfected cell culture. MW is radiolabeled Hpa II digest of pBR322 DNA. Lanes 1 and 2, control; lanes 3 and 4, 1 mM HU 20 minutes; lanes 5 and 6, 1 mM Hu 40 minutes; lanes 7 and 8, 1 mM HU 60 minutes.

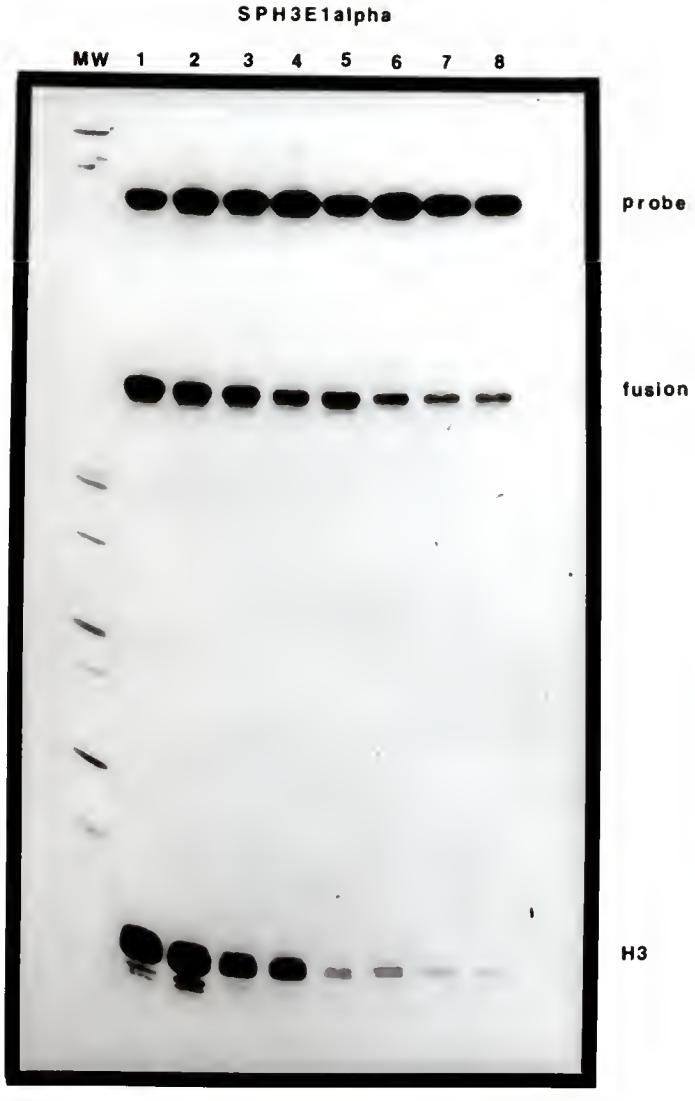
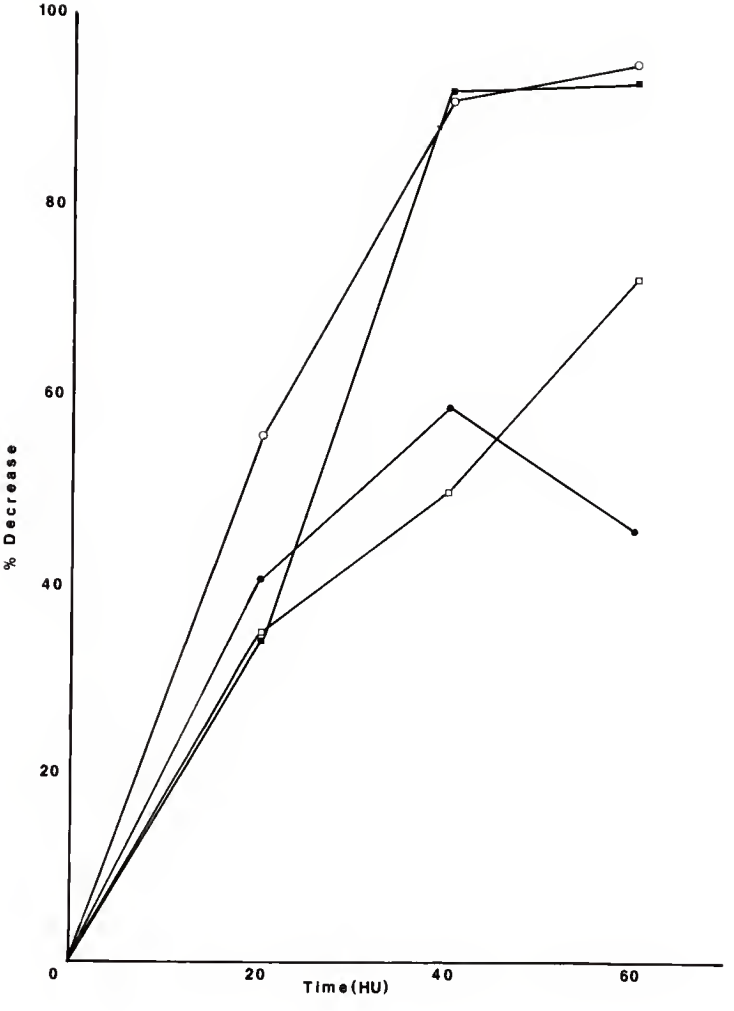

SPH3E1alpha
MW
1
2
3
4
5
6
7
8
probe
fusion
H3

Figure 4-10. Quantitation of SPH3E1, SPH3E1alpha, SPH3E1ATG$^-$ mRNA during hydroxyurea treatment.

The cellular levels of the signal peptide-histone fusion mRNAs and endogenous H3 histone mRNA during hydroxyurea treatment were determined by densitometric analysis of the S1 nuclease protection assays presented in Figures 4-7 to 4-9. Values are presented as percentage of control cells (●, SPH3E1 mRNA;□, SPH3E1alpha mRNA;■, SPH3E1ATG$^-$ mRNA;○, endogenous H3 histone mRNA).


100
80
60
40
20
0
% Decrease
20
40
60
Time(HU)

resulted in the destabilization of approximately 70% of SPH3E1alpha mRNA, a value which is intermediate to that observed for SPH3E1 mRNA and SPH3E1ATG$^-$ mRNA (Figure 4-9 lanes 1,2 and 7,8; Fig. 4-10). The degree to which the signal peptide-histone fusion mRNAs are degraded during inhibition of DNA synthesis is correlated with the extent to which the mRNA is associated with nonmembrane-bound polysomes. These results suggest that the subcellular localization of the histone mRNA plays a significant role in the coupling of its stability to DNA replication.

Discussion

To our knowledge, this represents the first demonstration that the addition of a signal peptide coding sequence to an mRNA that is normally translated on nonmembrane-bound polysomes results in the targeting of the chimeric message to membrane-bound polysomes in intact mammalian cells. The recognition of a prokaryotic signal peptide in a eukaryotic cell is supported by both *in vitro* and *in vivo* studies (Lingappa *et al.*, 1984; Talmadge *et al.*, 1980a; Talmadge *et al.*, 1980b). *Saccharomyces cerevisiae* has been shown to express the *E. coli* pBR325 beta-lactamase gene *in vivo* and to process the precursor protein to the enzymatically active, mature protein (Roggenkamp *et al.*, 1981). The processing of the bacterial pre-protein into the mature species has also been demonstrated *in vitro* using a

crude yeast extract (Roggenkamp et al., 1981). In addition, it has been reported by Weidmann et al. (1984) that E. coli plasmid pBR322 beta-lactamase mRNA that is synthesized and capped in vitro, when microinjected into Xenopus oocytes, is translated into protein that is ultimately secreted from the cell. These results suggest a common mechanism of signal peptide recognition among prokaryotic and eukaryotic organisms.

In an attempt to prevent the translocation of the signal peptide-histone fusion mRNA to membrane-bound polysomes, we initially disrupted the hydrophobic region of the signal peptide by site directed mutagenesis. This approach was based on previous studies demonstrating that bacterial secretory proteins that were mutated in the hydrophobic domain, either by insertion of charged amino acids or by deletion, accumulate in the cytoplasm of the bacterium (Emr et al., 1978; Bedouelle et al., 1980; Emr and Bassford, Jr., 1982). The incorporation of positively charged histidine residues in the hydrophobic domain of the signal peptide (SPH3E1alpha) however, resulted in only a partial block in the association of mutated signal peptide-histone fusion mRNA with membrane-bound polysomes (Figure 4-6). This result is not surprising in light of more recent studies on protein export in eukaryotes, which demonstrate that the relationship between the primary sequence of the signal peptide and its ability to function as a secretory

signal is quite variable and can withstand substantial insertion, substitution or deletion mutations (Kaiser and Botstein, 1986; Kaiser et al., 1987; Randall and Hardy, 1989). Subsequently, the signal peptide ATG translation start codon of SPH3E1 was altered by site directed mutagenesis which completely inactivated signal peptide function (Figure 4-6).

The relative stability of signal peptide-histone fusion mRNA (SPH3E1) during inhibition of DNA synthesis appears to be a result of the subcellular location of the mRNA. Destabilization of SPH3E1ATG$^-$ mRNA, which differs from SPH3E1 mRNA by a single nucleotide, with essentially the same kinetics and to the same extent as endogenous H3 histone mRNA indicates that SPH3E1 mRNA expresses the proper structural confirmation for recognition by the factors that mediate the selective destabilization of histone mRNA during inhibition of DNA synthesis.

The difference in SPH3E1 and SPH3E1ATG mRNA stability during inhibition of DNA synthesis may reflect a perturbation of protein structure due to the signal peptide at the N-terminus of SPH3E1. This possibility does not appear likely based on the observations that histone amino acid sequences are not required to target the mRNA for destabilization (Pandey and Marzluff, 1987) and SPH3E1alpha mRNA, which expresses the mutated form of the signal peptide, is moderately destabilized during inhibition of DNA synthesis (Figures 4-8 and 4-10).

These results indicate that the stability of SPH3E1 mRNA during inhibition of DNA replication is functionally related to the change in the subcellular location of the mRNA. The differential stability of SPH3E1 mRNA and SPH3E1ATG^{-} mRNA may be due to qualitative differences in the composition of membrane-bound and nonmembrane-bound polysomes; however, there is no previously reported evidence to support this possibility. Alternatively, the presence of the fusion mRNA on membrane-bound polysomes rather than nonmembrane-bound polysomes, where histone mRNA normally resides, may physically separate the message from the factors that are involved in the selective destabilization of histone mRNA during inhibition of DNA synthesis. These results are consistent with the hypothesis that the subcellular location of histone mRNA plays an important role in the posttranscriptional regulation of histone gene expression.

CHAPTER 5

DIFFERENTIAL ASSOCIATION OF MEMBRANE-BOUND AND NONMEMBRANE-BOUND POLYSOMES WITH CELL CYTOSTRUCTURE

Introduction

Localization of nearly all actively translated polyribosomes on the cytoskeletal structure indicates that polysomal mRNAs are not freely diffusible throughout the cytoplasm (Lenk et al., 1977; Cervera et al., 1981; Ornelles et al., 1986). This observation is further supported by subcellular fractionation studies that demonstrate the association of both "free" (nonmembrane-bound) and membrane-bound polysomal mRNAs with the cytoskeleton (Cervera et al., 1981; Jeffery, 1984; Bonneau et al., 1985).

Previous results indicate that the association of polysomes with the cytoskeleton is mediated in part by the mRNA, as treatments which dissociate the ribosomes from the mRNA, such as heat shock and high salt, release the ribosomal subunits into the soluble phase while the mRNA remains attached to the cytoskeleton (Lenk et al., 1977; Cervera et al., 1981; van Venrooij et al., 1981; Howe and Hershey, 1984). However, the region of the mRNA and the cytoskeletal elements to which it is attached are not known.

We present evidence here to support a model for multiple sites of attachment of eukaryotic mRNA to the cytoskeleton. Using cytochalasin D for the disruption of microfilaments, we have observed a differential release of specific mRNAs from the cytoskeleton. Further characterization of the association of mRNA with the cytoskeleton revealed an additional attachment site that is insensitive to cytochalasin D treatment and present only in membrane-bound polysomal mRNAs. This second attachment site is puromycin sensitive and appears to involve the association of the nascent polypeptide and/or ribosome with the remnant protein structure of the endoplasmic reticulum.

Results

Cytochalasin D Releases Poly A^- RNA from the Cytoskeleton

Disruption of actin fibers by cytochalasin D treatment results in the release of cytoplasmic poly A^+ RNA from the cytoskeleton in a dose dependent manner (Ornelles et al., 1986). To further understand the association of eukaryotic mRNAs with the cytoskeleton, we have studied the effects of cytochalasin D treatment on the release of specific mRNAs, including those that are non-polyadenylated. In addition we have examined and compared the cytoskeletal association of nonmembrane-bound and membrane-bound polysomal mRNAs.

Cell cycle dependent histone genes code for mRNAs that are capped, non-polyadenylated and predominantly associated

with the cytoskeleton (Jeffery, 1984; Stein et al., 1977; Adensik and Darnell, 1972; Zambetti et al., 1985). The distribution of histone mRNAs between the cytoskeletal and the soluble fractions was determined by Northern blot analysis before and after cytochalasin D treatment. As seen in Figure 5-1, H3 and H4 cell cycle dependent histone mRNAs are primarily associated with the cytoskeleton in untreated HeLa cell cultures (lanes 1 and 2). Quantitation of the autoradiograph in Figure 5-1 by scanning laser densitometry revealed that >90% of the histone mRNAs were associated with the cytoskeleton in control cell cultures (Table 5-1), which is consistent with previously reported values (Zambetti et al., 1985). Upon addition of cytochalasin D, histone mRNAs were released from the cytoskeleton into the soluble phase (Fig. 5-1, lanes 3-8). As shown in Table 5-1, less than 35% of the histone mRNAs remained associated with the cytoskeleton in HeLa cell cultures treated with 10 μg/ml cytochalasin D. Treatment with 40 μg/ml cytochalasin D resulted in the dissociation of >70% of the histone mRNAs from the cytoskeleton. The extent to which cytochalasin D releases non-polyadenylated histone mRNA from the cytoskeleton is similar to that reported for total, heterogeneous poly-A^+ RNA (Ornelles et al., 1986).

Membrane-Bound Polysomal RNA is not Released from the Cytoskeleton by Cytochalasin D Treatment

HLA-B7 is a class I histocompatibility antigen that is located on the cell surface (Robb et al., 1978). Previously, HLA-B7 mRNA was shown to be associated with the cytoskeleton and translated into protein on membrane-bound polysomes (Zambetti et al., 1985). As seen in Figure 5-1, HLA-B7 mRNA was almost exclusively associated with the cytoskeleton in control cells (lanes 1 and 2) and remained largely associated with the cytoskeleton in cytochalasin D treated cells (lanes 3-8). Approximately 98% of the HLA-B7 mRNA was associated with the cytoskeleton in untreated cell cultures (Table 5-1). In contrast to histone mRNA and total poly-A^+ RNA, only 11% of the HLA-B7 mRNA was released from the cytoskeleton in cell cultures treated with 10 μg/ml cytochalasin D (Table 5-1).

The retention of HLA-B7 mRNA on the cytoskeleton during cytochalasin D treatment may be a consequence of translating the mRNA on membrane-bound polysomes. To examine this possibility, the distribution of chorionic gonadotropin alpha mRNA within the cytoskeleton and soluble fractions was determined in cytochalasin D treated cells. Chorionic gonadotropin is a secreted, heterodimeric protein composed of alpha and beta subunits (Milsted et al., 1985). The alpha subunit is expressed in exponentially growing HeLa cells (Milsted et al., 1985), and Northern blot analysis of

Figure 5-1. Northern blot analysis of the cytoskeleton and soluble phase distribution of H3 histone, H4 histone and HLA-B7 mRNAs in cytochalasin D treated cells.

HeLa cells were treated with the indicated concentration of cytochalasin D for 20 minutes and cytoskeleton and soluble phase RNAs were isolated. Equal quantities of RNA per sample (10 μg/lane) were analyzed by Northern blot analysis as described in Chapter 2. Lanes: 1) Control/csk; 2) Control/sol; 3) CD [5 μg/ml]/csk; 4) CD [5 μg/ml]/sol; 5) CD [10 μg/ml]/csk; 6) CD [10 μg/ml]/sol; 7) CD [40 μg/ml]/csk; 8) CD [40 μg/ml]/sol.

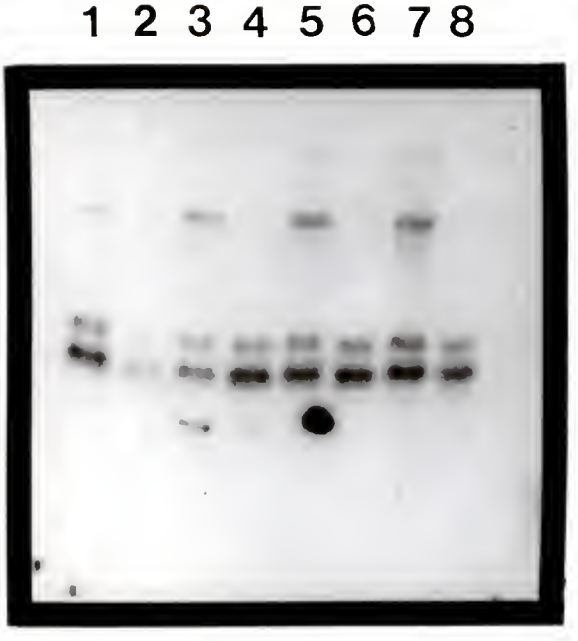

1 2 3 4 5 6 7 8
HLA-B7
H3
H4

Table 5-1. The percent of cytoskeleton and soluble phase associated mRNAs in cells treated with cytochalasin D.

		H3	H4	HLA-B7	hCG
Control	Csk	93%	96%	>98%	90%
	Sol	7%	4%	< 2%	10%
CD [5 μg/ml]	Csk	24%	20%	79%	69%
	Sol	76%	80%	21%	31%
CD [10 μg/ml]	Csk	33%	31%	87%	77%
	Sol	67%	69%	13%	23%
CD [40 μg/ml]	Csk	26%	20%	64%	41%
	Sol	74%	80%	36%	59%

The densitometric results from Figure 5.1 (hCG data not shown) were normalized for the yield of RNA from the cytoskeleton and soluble fractions of each cell sample (note: equal quantities of cytoskeleton and soluble RNAs were analyzed which does not take into consideration the unequal distribution of RNA within these fractions or the changes that occur during cytochalasin D treatment). The values are presented as percent distribution of mRNA between the cytoskeleton and soluble fractions.

subcellular fractions demonstrated that approximately 75% of the chorionic gonadotropin alpha (hCGα) mRNA was associated with membrane-bound polysomes (autoradiograph not shown). Analogous to HLA-B7 mRNA, hCGα mRNA was not fully released from the cytoskeleton in cytochalasin D treated cells; greater than 90% of the gonadotropin mRNA was associated with the cytoskeleton in control cells and approximately 77% remained associated with the cytoskeleton in cells treated with 10 μg/ml cytochalasin D (autoradiograph not shown; data summarized in Table 5-1).

Nonmembrane-Bound Polysomal mRNAs are Released from the Cytoskeleton by Cytochalasin D Treatment

Previous results have demonstrated that histone mRNAs are predominantly translated on cytoskeleton associated, nonmembrane-bound polysomes (Zambetti et al., 1985) and as described above, are released from the cytoskeleton into the soluble phase by cytochalasin D treatment. As an additional control for studying the release of nonmembrane-bound polysomal RNAs from the cytoskeleton by cytochalasin D treatment, we analyzed the distribution of c-fos mRNA between the cytoskeleton and soluble fractions from control and cytochalasin D treated cell cultures. The c-fos protein is localized within the nucleus (Curran et al., 1984) and therefore, according to the signal hypothesis, should be synthesized on nonmembrane-bound polysomes (Blobel and

Dobberstein, 1975). Subcellular fractionation and Northern blot analysis revealed that c-fos mRNA is predominantly associated with nonmembrane-bound polysomes (data not shown), and that approximately 80% of the c-fos mRNA is associated with the cytoskeleton (Figure 5-2, lanes 1 and 2; Table 5-2). Cytochalasin D as well as puromycin, CD/puromycin and CD/cycloheximide dramatically increased c-fos mRNA levels, and the newly synthesized c-fos mRNA partitioned into both the cytoskeletal and soluble fractions in a manner that reflects the drug treatment (Figure 5-2, lanes 3-10; Table 5-2). Less than 30% of the c-fos mRNA remained associated with the cytoskeleton in cytochalasin D [10 μg/ml] treated cells (Table 5-2).

Membrane-Bound Polysomal mRNAs Express Multiple Cytoskeleton Attachment Sites

Retention of membrane-bound polysomal mRNAs on the cytoskeleton in cytochalasin D treated cells suggests that this class of polysomal mRNA may contain additional cytoskeleton attachment sites. Cell surface proteins, such as HLA-B7 and chorionic gonadotropin, are generally synthesized on membrane-bound polysomes that are complexed with the endoplasmic reticulum (for review see Emr et al., 1980). The nature of the interaction between the polysomes and the endoplasmic reticulum appears to be mediated in part by the nascent polypeptide that is inserted into the

Figure 5-2. Northern blot analysis of the cytoskeleton and soluble phase distribution of c-fos mRNA in cytochalasin D, puromycin, CD/puro, and CD/cycloheximide treated cells.

HeLa cells were cultured in the presence of the drugs and fractionated into cytoskeleton and soluble phases as described in the Chapter 2. Equal quantities of RNA (10 μg/sample) from each fraction were assayed for c-fos mRNA content by Northern blot analysis. Lanes: 1) Control/csk; 2) Control/sol; 3) CD/csk; 4) CD/sol; 5) Puro/csk; 6) Puro/sol; 7) CD/puro/csk; 8) CD/puro/sol; 9) CD/cyclo/csk; 10) CD/cyclo/sol.

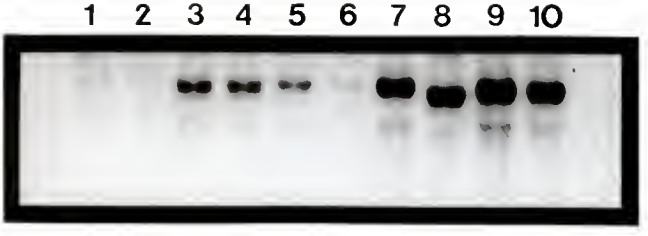

1 2 3 4 5 6 7 8 9 10
c-Fos

Table 5-2. Percent distribution of nonmembrane-bound and membrane-bound polysomal RNAs in the Csk and Sol fractions from CD, Puro, CD/puromycin and CD/cycloheximide treated cells.

		H3/H4	HLA-B7	hCG	fos
Control	CSK	87% (±5)	97% (±1)	96% (±1)	80%
	SOL	13%	3%	4%	20%
CD	CSK	18% (±4)	75% (±6)	72% (±6)	29%
	SOL	82%	25%	28%	71%
Puro	CSK	79% (±7)	79% (±9)	85% (±5)	83%
	SOL	21%	21%	15%	17%
CD/Puro	CSK	18% (±4)	25% (±3)	26% (±3)	20%
	SOL	82%	75%	74%	80%
CD/Cyclo	CSK	19% (±8)	81% (±2)	52% (±10)	40%
	SOL	81%	19%	48%	60%

The Csk and Sol distribution of c-fos mRNA during CD, puro, CD/puro and CD/cyclo treatment was quantitated by normalizing the densitometric results from the autoradiogram presented in Figure 5-2 for the yield of RNA in each fraction. The Csk and Sol distribution of nonmembrane-bound and membrane-bound polysomal mRNAs was quantitated directly from the densitometric analysis. The numbers in brackets indicate standard error.

membrane of the endoplasmic reticulum. In addition, the nascent polypeptide is complexed with the proteins involved in the translocation of the exported protein from the cytoplasm into the lumen of the endoplasmic reticulum. During the isolation of the cytoskeleton nearly all the lipid components but not the protein substructure of the endoplasmic reticulum membrane are removed by detergent extraction (Lenk et al., 1977). The cytoskeleton attachment site associated with membrane-bound polysomes, which is insensitive to cytochalasin D, may be the nascent polypeptide complexed with the remnant protein structure of the endoplasmic reticulum.

To test the possibility that the nascent polypeptide serves as an additional anchor for the attachment of mRNA to the cytoskeleton, we co-treated HeLa cells with cytochalasin D and puromycin. Puromycin inhibits protein synthesis by interrupting polypeptide chain formation which results in the release of the nascent polypeptide and ribosomal subunits from the mRNA. The distribution of membrane-bound and nonmembrane-bound polysomal RNA within the cytoskeleton and soluble fractions was then determined by Northern blot analysis.

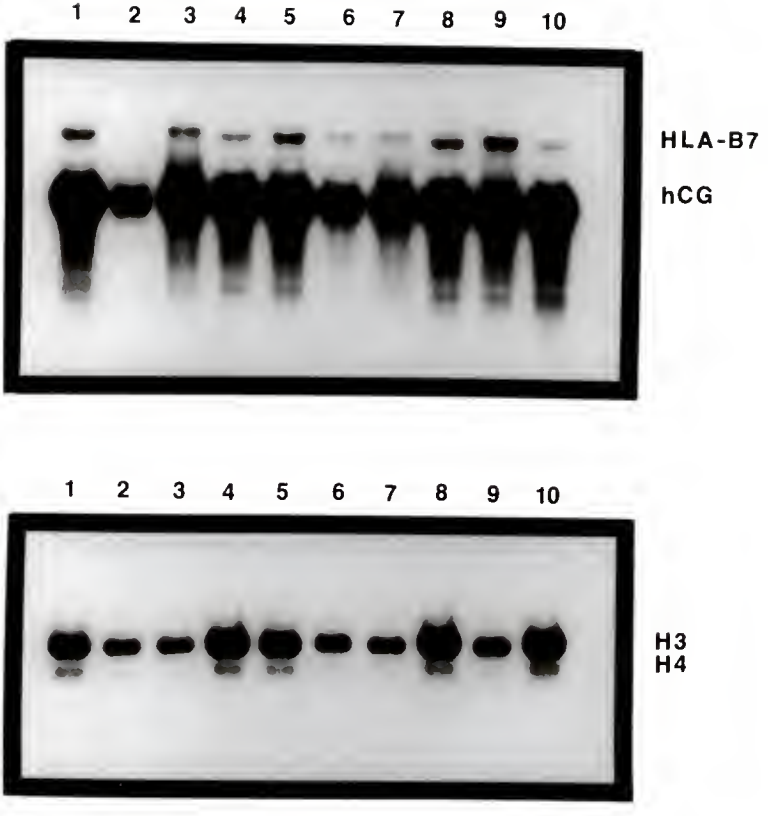
As seen in Figure 5-3, treatment of HeLa cells with cytochalasin D and puromycin released membrane-bound polysomal mRNAs from the cytoskeleton (lanes 7 and 8). Approximately 25% of HLA-B7 mRNA and 20% of hCGα mRNA

remained associated with the cytoskeleton in cytochalasin D treated cells that were subsequently treated with puromycin (Table 5-2). In contrast, more than 75% of the HLA-B7 mRNA and 72% of the hCGα mRNA remained associated with the cytoskeleton in HeLa cells treated with cytochalasin D alone (Table 5-2). The release of membrane-bound polysomal mRNAs from the cytoskeleton in cytochalasin D and puromycin treated HeLa cells was not a direct result of the inhibition of protein synthesis. Cytochalasin D treatment followed by the inhibition of protein synthesis with cycloheximide, a compound that preserves the polysomal structure, failed to release HLA-B7 mRNA from the cytoskeleton (Figure 5-3, lanes 9 and 10). Greater than 80% of the HLA-B7 mRNA was associated with the cytoskeleton in cytochalasin D and cycloheximide treated cells (Table 5-2). The results were not as dramatic for the hCGα mRNA (Figure 5-3, lanes 9 and 10). Approximately 52% of hCGα mRNA was associated with the cytoskeleton in cells treated with both cytochalasin D and cycloheximide (Table 5-2).

The release of HLA-B7 mRNA and hCGα mRNA from the cytoskeleton in cytochalasin D and puromycin treated HeLa cells was not due solely to the inhibition of protein synthesis by puromycin. As previously reported, the dissociation of polysomes with high salt or heat shock resulted in the release of the ribosomal subunits into the soluble phase without affecting the attachment of poly A^+

Figure 5-3. Northern blot analysis of the cytoskeleton and soluble phase distribution of nonmembrane-bound and membrane-bound polysomal mRNAs in cytochalasin D, puromycin, CD/puro and CD/cycloheximide treated cells.

HeLa cells were cultured in the presence of the drugs and fractionated into cytoskeleton and soluble phases as described in Chapter 2. Equal volumes of cytoskeleton and soluble phase RNA from each cell culture were assayed for H3 histone, H4 histone, HLA-B7 and chorionic gonadotropin mRNAs by Northern blot analysis. Upper panel, probed for HLA-B7 and chorionic gonadotropin mRNA, and lower panel, probed for H3 and H4 histone mRNA. Upper and lower panels represent the same Northern filter. Lanes: 1) Control/csk; 2) Control/sol; 3) CD/csk; 4) CD/sol; 5) Puro/csk; 6) Puro/sol; 7) CD/puro/csk; 8) CD/puro/sol; 9) CD/cyclo/csk; 10) CD/cyclo/sol.


1 2 3 4 5 6 7 8 9 10
HLA-B7
hCG
1 2 3 4 5 6 7 8 9 10
H3
H4

mRNA to the cytoskeleton (Cervera et al., 1981; Howe and Hershey, 1984). Consistent with this finding, 79% of HLA-B7 mRNA and 85% of hCGα mRNA remained attached to the cytoskeleton in puromycin treated HeLa cells (Table 5-2). In addition, 79% of the H3 and H4 histone poly A^- mRNA and 83% of the c-fos mRNA remained associated with the cytoskeleton in HeLa cells treated with puromycin (Table 5-2).

Discussion

There is growing support for the hypothesis that the cytoskeleton of eukaryotes is more than a structural scaffold. Evidence is accumulating to suggest that the cytoskeleton is a dynamic structure that plays an integral role in the process of protein synthesis. In addition, the cytoskeleton may influence the posttranscriptional regulation of eukaryotic genes by compartmentalizing mRNAs within the cytoplasmic space. It is therefore of importance to study the interactions of eukaryotic mRNAs with the cytoskeleton.

The experiments presented here were designed to examine the association of specific mRNAs with the cytoskeleton. As described previously and confirmed in this study, histone mRNA and HLA-B7 mRNA were found predominantly associated with the cytoskeleton (Zambetti et al., 1985). In addition, hCGα and c-fos mRNAs were also localized in the cytoskeleton fraction. Recently it was shown that the association of poly

A^+ RNA with the cytoskeleton could be disrupted by perturbing the microfilaments with cytochalasin D in HeLa cells (Ornelles et al., 1986). Consistent with these results, cytochalasin D treatment readily releases c-fos mRNA and poly-A^- histone mRNA from the cytoskeleton into the soluble phase. Surprisingly, HLA-B7 mRNA and hCGα mRNA were only partially released from the cytoskeleton in cytochalasin D treated HeLa cells.

The inefficiency of cytochalasin D in releasing HLA-B7 and hCGα mRNA from the cytoskeleton appears to be a result of the association of these mRNAs with membrane-bound polysomes. Dissociation of the polysomes with puromycin in the presence of cytochalasin D releases HLA-B7 and hCGα mRNA from the cytoskeleton. In contrast, cycloheximide, an inhibitor of protein synthesis that preserves polysomal structure, fails to release HLA-B7 mRNA from the cytoskeleton in cytochalasin D treated cells. These results suggest that membrane-bound polysomal mRNAs are attached to the cytoskeleton in at least two different ways. The first cytoskeleton attachment site is through the mRNA/mRNP itself and is sensitive to cytochalasin D treatment. This site is most likely common to all cytoskeletal associated mRNAs in HeLa cells and has been previously observed for poly A^+ RNA by Ornelles et al. (1986). The second cytoskeletal attachment site which is characteristic of membrane-bound polysomes appears to be mediated by the nascent polypeptide

and/or ribosome that is complexed with the endoplasmic reticulum. The possibility that the nascent polypeptide serves as an additional cytoskeleton attachment site for membrane-bound polysomes is supported by the observation that nascent polypeptides undergoing translocation into the lumen of the endoplasmic reticulum are resistant to protease digestion following detergent extraction (Connolly et al., 1989). The protection of the nascent polypeptides from protease digestion, in the absence of membranes, appears to be due to the association of the polypeptide with the proteins of the translocation apparatus of the endoplasmic reticulum.

To release membrane-bound polysomal mRNAs efficiently from the cytoskeleton, both cytoskeletal attachment sites must be disrupted. Dissociation of the polysomes with puromycin alone fails to release membrane-bound polysomal mRNA from the cytoskeleton. In this case, the mRNA remains associated with the cytoskeleton through the cytochalasin D sensitive site even though the polysomes are no longer anchored to the endoplasmic reticulum. Consistent with this reasoning, cytochalasin D treatment alone results in only a partial release of membrane-bound polysomal mRNA from the cytoskeleton. The partial release of membrane-bound polysomal mRNA from the cytoskeleton in cytochalasin D treated cells is most likely a result of the release of mRNA from the cytochalasin D sensitive cytoskeleton attachment

site coupled with a partial "run off" of the polysomes from the mRNA, which in effect dissociates the nascent polypeptide from the mRNA.

In addition to the differential association of membrane-bound and nonmembrane-bound polysomes with the cytoskéleton, there also appears to be differences in the cytoskeletal interaction of individual species of mRNA. As seen in Table 5-2, hCGα mRNA and HLA-B7 mRNA are released from the cytoskeleton to the same extent during CD and puromycin cotreatment, which is consistent with the mechanism for the association of membrane-bound polysomes with the cytoskeleton as described above. However, CD and cycloheximide cotreatment releases a larger percentage of hCGα mRNA from the cytoskeleton than CD treatment alone. In contrast, HLA-B7 mRNA is associated with the cytoskeleton to the same extent or greater in cytochalasin D and cycloheximide co-treated cells than HeLa cells treated with CD alone. Furthermore, CD and cycloheximide cotreatment readily dissociates histone mRNA from the cytoskeleton whereas c-fos mRNA is only partially affected. These results suggest that multiple mechanisms are operative for the association of individual mRNAs and/or polysomes with the cytoskeleton and may reflect differences in the structural environment of the cell.

The possibility that the structural environment of the cell may affect cytoskeletal-mRNA interactions is supported

by the apparent cell type specific differences that are observed for the components of the cytoskeleton that are involved in the attachment of mRNA and polysomes. Adams et al. (1983) found that polyribosomes in rat liver cells are associated with the microfilaments and are released from the cytoskéleton by deoxyribonuclease I (DNase I), an enzyme that can depolymerize actin. Pretreatment of the rat liver cells with phalloidin, a compound that stabilizes actin filaments, abrogates the DNase I effects on the polyribosome-cytoskeleton interactions (Adams et al., 1983). Consistent with this result, poly A^+ RNA, translation factors and nearly 100% of the polysomes are preferentially associated with the cytoskeleton in HeLa cells and can be released into the soluble phase by the disruption of the microfilaments with cytochalasin B or cytochalasin D (Lenk et al., 1977; Howe and Hershey, 1984; Ornelles et al., 1986). In contrast, Jeffery (1984) reported that the association of actin mRNA and histone mRNA with the cytoskeleton in ascidian eggs is not affected by cytochalasin B treatment, which suggests that the association is independent of the microfilaments. Furthermore, disruption of the microfilaments by cytochalasin B treatment does not release poly A^+ histone H4 mRNA from the cytoskeleton in L6 rat myoblast cells (Bagchi et al., 1987).

The association of mRNA with the cytoskeleton also appears to change during differentiation in certain cell types (Toh et al., 1980). Microscopic analysis of fetal rat lung fibroblast cells indicates that polyribosomes are aligned in linear arrays along the actin-like stress fibers (Toh et al., 1980). These polysomes are difficult to visualize after disruption of the microfilaments by cytochalasin B treatment and are presumed to be released from the cytoskeleton into the soluble phase. Polysomes in the more differentiated fetal lung fibroblasts are associated with the endoplasmic reticulum and are no longer affected by cytochalasin B treatment (Toh et al., 1980). These results are consistent with the possibility that the association of polysomes with the cytoskeleton changes during differentiation.

In conclusion, the results presented here provide evidence for heterogeneity in the interactions of mRNA with the cytoskeleton in HeLa cells. Membrane-bound and non-membrane-bound polysomes are differentially associated with the cytoskeleton. Studies on the association of mRNA with the cytoskeleton must take into consideration these differences when interpreting which elements are involved in the cytoskeletal attachment of mRNA. Failure to release mRNA from the cytoskeleton with cytochalasins may only suggest that additional cytoskeletal attachment sites are involved. The differential association of mRNA with the cytoskeleton

may play an important part in the subcellular localization of messenger RNA, which may ultimately affect posttranscriptional regulation. At this time, we can not rule out the possibility that mRNAs localized in different regions of the cytoplasm are associated with the cytoskéleton by different mechanisms. Additional studies are currently underway to define further the association of mRNA with the cytoskeleton and what influence this structure has on the regulation of eukaryotic gene expression.

CHAPTER 6

HETEROGENEOUS PATTERN FOR CYTOSKELETAL-mRNA INTERACTIONS

Introduction

In the preceding chapter we presented evidence indicating that eukaryotic mRNAs are associated with the cytoskeleton in a heterogeneous manner. Disruption of the microfilaments by cytochalasin D treatment readily releases nonmembrane-bound polysomes from the cytoskeleton into the soluble phase, whereas membrane-bound polysomes remain attached to the cytoskeleton. The cytoskeletal association of membrane-bound polysomal mRNAs occurs through at least two distinct sites: a cytochalasin D sensitive site and a puromycin sensitive site, which is the interaction of the nascent polypeptide/polysome with the protein substructure of the endoplasmic reticulum (Chapter 5). To release membrane-bound polysomes from the cytoskeleton, both attachment sites must be disrupted as seen during cytochalasin D and puromycin cotreatment. Previously, we have demonstrated that a signal peptide-histone fusion mRNA (SPH3E1) is targeted from nonmembrane-bound polysomes, the natural site of histone protein synthesis, to membrane-bound polysomes (Chapter 4). Relocating the histone mRNA within

the cell enabled us to examine mRNA-cytoskeleton interactions in more detail. The results presented here indicate that the signal peptide-histone fusion mRNA is associated with the cytoskeleton in a manner unlike that reported for membrane-bound and nonmembrane-bound polysomes. These results are consistent with the existence of a cytoskeletal attachment element within the mRNP or mRNA primary sequence and provide additional evidence for heterogeneity in mRNA-cytoskeleton interactions.

Results

To investigate further the mechanism of mRNA attachment to the cytoskeleton, we have studied and compared the cytoskeletal association of endogenous nonmembrane-bound polysomal histone mRNA with that of membrane-bound polysomal, signal peptide-histone chimeric mRNA. In Chapter 4, we have described the construction of a signal peptide-histone chimeric gene (SPH3E1) that when transfected into HeLa cells is transcribed and the resulting chimeric histone mRNAs are targeted to membrane-bound polysomes (Zambetti *et al.*, 1986). Although the signal peptide fused to the histone gene is derived from the beta-lactamase gene of the *E. coli* plasmid pBR322, it is functional in HeLa cells (Chapter 4). Figure 4-5 schematically diagrams endogenous H3 histone and the signal peptide-histone (SPH3E1) mRNAs.

Monoclonal HeLa cell lines expressing the chimeric gene were fractionated into cytoskeleton and soluble phases as described by Cervera et al. (1981). Subsequently, RNA from each fraction was isolated and SPH3E1 chimeric mRNA and endogenous H3 histone mRNA content was determined by S1 nuclease protection analysis (Chapter 2). As seen in Figure 6-1 and Table 6-1, both endogenous histone and SPH3E1 mRNAs are predominantly associated with the cytoskeleton in control cells (92% and 93%, respectively). Cytochalasin D treatment [10 μg/ml] brought about a limited release of SPH3E1 mRNA from the cytoskeleton as expected for a membrane-bound polysomal mRNA. Only 15% of SPH3E1 mRNA compared to 40% of endogenous histone mRNA was dissociated from the cytoskeleton in CD treated cells. As described above, dissociation of the ribosomes by puromycin during cytochalasin D treatment is necessary for the efficient release of membrane-bound polysomal mRNAs from the cytoskeleton. Surprisingly, CD and puromycin cotreatment also failed to release the SPH3E1 mRNA from the cytoskeleton. Greater than 70% of the signal peptide-histone chimeric mRNA and less than 26% of endogenous H3 histone mRNA remained associated with the cytoskeleton after CD and puromycin cotreatment. The selective retention of the chimeric mRNA on the cytoskeleton during CD and puromycin cotreatment is demonstrated by the marked increase in the intensity of the signal obtained for the same quantity of

Figure 6-1. Cytochalasin D and puromycin cotreatment does not release SPH3E1 or SPH3E1ATG$^-$ mRNA from the cytoskeleton.

Clonal cell lines expressing SPH3E1 or SPH3E1ATG$^-$ mRNA were treated with 10 μg/ml cytochalasin D, 0.4 mM puromycin or 10 μg/ml cytochalasin D and 0.4 mM puromycin. The distribution of endogenous histone and fusion mRNAs within the cytoskeleton and soluble phase fractions was determined by S1 nuclease protection analysis as described in Chapter 2. Analysis of a) SPH3E1 and SPH3E1ATG$^-$ mRNAs and b) endogenous H3 histone mRNA. Lanes 1-8 represent RNA samples isolated from SPHE1 expressing cells and lanes 9-16 represent RNA samples isolated from SPH3E1ATG$^-$ cell cultures. Lanes 1 and 9, control-csk RNA; lanes 2 and 10, control-sol RNA; lanes 3 and 11, CD-csk RNA; lanes 4 and 12, CD-sol RNA; lanes 5 and 13, puro-csk RNA; lanes 6 and 14, puro-sol RNA; lanes 7 and 15, CD/puro-csk RNA; lanes 8 and 16, CD/puro-sol RNA.

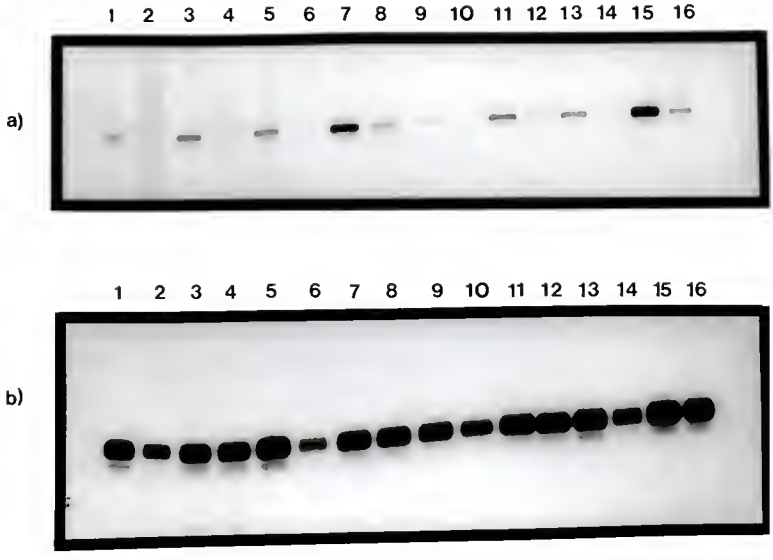

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
a)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
b)

Table 6-1. The percent of cytoskeleton associated mRNAs isolated from cytochalasin D, puromycin and CD/puromycin treated cells.

	Control	CD	Puro	CD/Puro
A) SPH3E1	>95%	93%	--	62%
Endogenous H3	81%	31%	--	27%
B) SPH3E1	93%	79%	94%	72%
hCG alpha	98%	81%	95%	18%
HLA-B7	99%	84%	93%	28%
Endogenous H3	92%	46%	90%	26%
C) SPH3E1ATG$^-$	>90%	87%	>89%	77%
hCG alpha	98%	81%	89%	29%
HLA-B7	97%	89%	88%	35%
Endogenous H3	78%	56%	79%	24%

The densitometric results from autoradiographs represented in Figures 6-2 to 6-4 (A to C, respectively) were corrected for the total yield of RNA from each fraction (note: equal quantities of cytoskeleton and soluble RNAs were analyzed in S1 and northern assays which does not take into consideration the unequal distribution of RNA within these fractions or the changes that occur during CD and/or puromycin treatment).

RNA in this sample compared with the control cytoskeleton sample (Figure 6-1, lane 1 compared to lane 7). The inability of CD and puromycin to release SPH3E1 chimeric mRNA efficiently from the cytoskeleton is not unique to the monoclonal cell line and is also observed in a polyclonal cell population (Figure 6-2, Table 6-1). In this case, 62% of the endogenous H3 histone mRNA and less than 5% of the SPH3E1 mRNA is released from the cytoskeleton during cytochalasin D treatment. In CD and puromycin co-treated polyclonal cell cultures approximately 27% of the endogenous histone mRNA and 62% of the SPH3E1 mRNA remain associated with the cytoskeleton.

To investigate whether the failure to release the signal peptide-histone mRNA from the cytoskeleton with cytochalasin D and puromycin was related to the efficiency of the drug treatments, we studied the association of endogenous membrane-bound polysomal mRNAs with the cytoskeleton. The mRNAs coding for HLA-B7, a class I cell surface histocompatibility antigen and chorionic gonadotropin alpha (hCGα), a secreted protein, are translated on membrane-bound polysomes and well represented in HeLa cells. The distribution of HLA-B7 and hCGα mRNA in the cytoskeleton and soluble samples used in Figure 6-1 was determined by Northern blot analysis (Figure 6-3 and Table 6-1). As expected for membrane-bound polysomal mRNAs, HLA-B7 and hCGα mRNAs were not released by cytochalasin D treatment

Figure 6-2. The wild type signal peptide-histone fusion mRNA is not released from the cytoskeleton in CD and puromycin co-treated polyclonal HeLa cell cultures.

Polyclonal cell cultures expressing the SPH3E1 fusion gene were treated with cytochalasin D and puromycin for 20 minutes. Cytoskeleton and soluble RNAs were isolated and subjected to S1 nuclease protection analysis as described in Chapter 2. Lane 1, control-csk; lane 2, control-sol; lane 3, CD [10 μg/ml]-csk; lane 4, CD [10 μg/ml]-sol; lane 5, CD [30 μg/ml]-csk; lane 6, CD [30 μg/ml]-sol; lane 7, CD [10 μg/ml] and puro [0.4 mM]-csk; and lane 8, CD [10 μg/ml] and puro [0.4 mM]-sol. MW is radiolabeled Hpa II digest of pBR322.

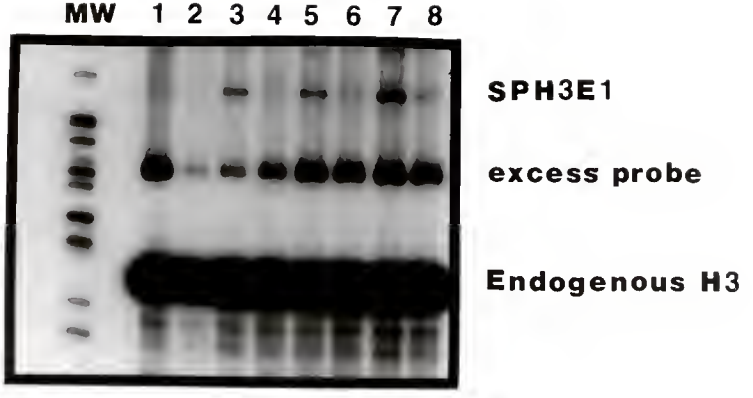
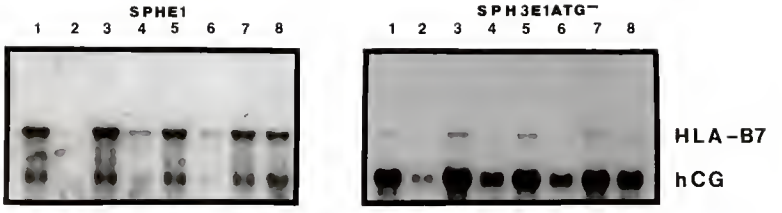

MW 1 2 3 4 5 6 7 8
SPH3E1
excess probe
Endogenous H3

Figure 6-3. Endogenous membrane-bound polysomal mRNAs are released from the cytoskeleton during cotreatment with CD and puromycin in SPH3E1 and SPH3E1ATG$^-$ expressing cell cultures.

Cytoskeleton and soluble RNAs (10 μg/sample) were assayed by Northern blot analysis for HLA-B7 mRNA and chorionic gonadotropin mRNA content as described in Chapter 2. Lane 1, control-csk; lane 2, control-sol; lane 3, CD-csk; lane 4, CD-sol; lane 5, puro-csk; lane 6, puro-sol; lane 7, CD/puro-csk; and lane 8, CD/puro-sol.


SPHE1
1 2 3 4 5 6 7 8
SPH3E1ATG⁻
1 2 3 4 5 6 7 8
HLA-B7
hCG

alone. Over 80% of these mRNAs were associated with the cytoskeleton after CD treatment. In contrast, less than 28% of the hCGα and HLA-B7 mRNAs remained associated with the cytoskeleton after CD and puromycin cotreatment indicating that the drug treatments were effective.

The association of the signal peptide-histone mRNA with the cytoskeleton in CD and puromycin co-treated cells suggests the existence of a cytoskeleton attachment site that is distinct from the site associated with endogenous histone mRNA and from other membrane-bound polysomes. These results indicate that the site is independent of the ribosomes and the nascent polypeptide and maybe a property of the nucleotide sequence and/or proteins that interact with the mRNA itself. Alternatively, the SPH3E1 chimeric mRNA may be efficiently translated and therefore support re-initiation of translation even in the presence of cytochalasin D and puromycin (note: endogenous histone mRNAs are efficiently translated; Stahl and Gallwitz, 1977). This would result in the synthesis of a portion of the signal peptide which could then serve as an anchor to the cytoskeletal structure as if the cells were treated with cytochalasin D alone.

To address this possibility, we have studied the cytoskeletal association of a mutated signal peptide-histone chimeric mRNA (SPH3E1ATG$^-$). Using site directed mutagenesis (Zoller and Smith, 1983) the ATG translation start codon for

the signal peptide of SPH3E1 was changed to TTG (Figure 4-5). Translation should therefore bypass this altered start codon and initiate at the normal ATG codon in the histone coding region, resulting in the synthesis of histone protein without the signal peptide sequence. Initial studies using a HeLa mónoclonal cell line expressing the SPH3E1ATG⁻ gene demonstrated the localization of SPH3E1ATG⁻ mRNA on nonmembrane-bound polysomes, which indicates that in vivo the signal peptide was not synthesized (Fig. 4-6). As seen in Figure 6-1, greater than 90% of SPH3E1ATG⁻ mRNA is associated with the cytoskeleton in the control cell culture. Cytochalasin D and puromycin cotreatment effectively released hCGα and HLA-B7 mRNA from the cytoskeleton with little or no effect on the association of the SPH3E1ATG⁻ mRNA with the cytoskeleton (Figures 6-1 and 6-3, Table 6-1). Approximately 77% of SPH3E1ATG⁻ mRNA and less than 35% of HLA and hCGα mRNA remained attached to the cytoskeleton under these conditions. Regardless of whether the SPH3E1ATG⁻ mRNA could be translated in the presence of cytochalasin D and puromycin, no signal peptide would be synthesized to anchor the message to the cytoskeleton. This result further supports the proposal that the nucleotide sequences coding for the signal peptide express a cytoskeleton attachment site which is distinct from the cytoskeletal attachment site of endogenous histone mRNA.

Discussion

The data presented in this chapter demonstrate that the signal peptide-histone fusion mRNAs (SPH3E1 and SPH3E1ATG$^-$) express a cytoskeleton attachment site that is not associated with histone mRNA, HLA-B7 mRNA or heterogeneous poly-A$^+$ mRNA (Chapter 5; Ornelles et al., 1986). It is not known whether the cytoskeleton attachment site unique to SPH3E1 and SPH3E1ATG$^-$ mRNA is due directly to the primary nucleotide sequences coding for the signal peptide, an alteration in the overall conformation of the mRNA or the proteins associated with the mRNP.

The elements of the cytoskeleton that are involved in the attachment of SPH3E1 mRNA and SPH3E1ATG$^-$ during cytochalasin D and puromycin cotreatment have not been identified. Association of the signal peptide-histone fusion mRNAs with the cytoskeleton during cytochalasin D and puromycin cotreatment indicates that the attachment site is independent of intact microfilaments. In addition, the cytochalasin D and puromycin insensitive cytoskeleton attachment site does not directly involve the microtubules since this cytoskeletal component is removed during the fractionation procedure (Lenk et al., 1977). The signal peptide-histone fusion mRNAs may be associated with the intermediate filaments of the cytoskeleton. Previous studies on other cell types indicate that mRNPs are associated with the cytoskeleton through the intermediate filaments

(Jeffery, 1984; Bagchi et al., 1987). Furthermore, prosomes, which are small ribonucleoprotein complexes that are associated with translationally inactive mRNAs, are attached to the cytokeratin-intermediate filaments in HeLa cells (Grossi de Sa et al., 1988). The role of the intermediate filaments in the attachment of the chimeric mRNAs to the cytoskeleton remains to be determined.

In Chapter 5, we demonstrated that the signal peptide-histone fusion mRNA (SPH3E1) is relatively stable, as compared to endogenous H3 histone mRNA, during inhibition of DNA synthesis. The uncoupling of signal peptide-histone fusion mRNA stability from DNA synthesis does not appear to be a consequence of its association with the cytoskeleton through the cytochalasin D and puromycin insensitive site. The mutated signal peptide-histone fusion mRNA (SPH3E1ATG$^-$) is efficiently destabilized during inhibition of DNA synthesis and most likely expresses the same CD and puromycin insensitive cytoskeletal attachment site as SPH3E1 mRNA.

In summary, a cytoskeletal attachment site has been detected which is associated with a signal peptide-histone chimeric mRNA. This site appears to be a direct or indirect result of the nucleotide sequences coding for the signal peptide. Studies on deletion and point mutants of the signal peptide-histone chimeric mRNAs should prove useful in providing insight into the elements involved in the attachment of the mRNA to the cytoskeleton.

CHAPTER 7

THE INFLUENCE OF THE CYTOSKELETON ON THE REGULATION OF c-Fos GENE EXPRESSION

Introduction

Modifications in gene expression is central to proliferation and differentiaton of eukaryotic cells. These processes are tightly regulated and dependent in part on the precise expression of proto-oncogenes (for review see Ohlsson and Pfeifer-Ohlsson, 1987; Bishop, 1983). The proliferative response of quiescent fibroblasts to serum or growth factor stimulation is accompanied by a rapid and transient induction of the c-fos proto-oncogene (Cochran et al., 1984; Greenberg et al., 1984; Curran et al., 1985). Within minutes of the addition of serum or growth factors to quiescent fibroblasts the c-fos gene is transcribed and c-fos cytoplasmic mRNA levels rapidly increase. Generally, the expression of the c-fos gene during serum/growth factor induction is maximal after 30 minutes and returns to pre-stimulatory levels within 1-2 hours. While the exact function of the c-fos protein is unknown, recent evidence suggests that this nuclear protein is part of a DNA binding

complex that may influence the transcription of specific genes (Curran et al., 1984; Sytoyama et al., 1986; Distel et al., 1987; Rauscher et al., 1988).

Signal transduction models describing the induction of c-fos gene expression due to the binding of growth factor(s) at the plasma membrane must include a mechanism for relaying the stimulatory signal from the cell surface, through the cytoplasm and into the nucleus. Existing within the cytoplasm of the cell is an extensive and intricate protein scaffold referred to as the cytoskeleton (for review see Nielsen et al., 1983). In addition to its structural properties the cytoskeleton also appears to function in cell shape, cell motility, the intracellular transport of macromolecules and translation of mRNA into protein. Furthermore, the cytoskeleton may influence the regulation of gene expression. Suspension of anchorage dependent mouse 3T6 fibroblasts induces a morphological change in cell shape and the cytoskeleton with a concomitant reduction in DNA, RNA and protein synthesis (Benecke et al., 1978; Farmer et al., 1983). Reattachment of these cells to a solid support restores cell shape and cytoskeleton structure as well as DNA, RNA and protein synthesis. In addition, disruption of the microfilaments of the cytoskeleton with cytochalasin D in HEp-2 cells results in the enhanced expression of beta and gamma actin protein synthesis (Tannenbaum and Godman, 1983). Removal of the drug restores the structure of the cytoskeleton and returns actin synthesis to normal levels.

The cytoskeleton also appears to play a role in the transition between proliferation and the expression of differentiated phenotypes. For example, normal B cells can be induced to proliferate by co-stimulation with anti-immunoglobulin and B cell growth factor (Yoshizaki et al., 1982). Cytochalasin D (CD) can substitute for B cell growth factor and provide the signal, presumably through the perturbation of the cytoskeleton, to induce proliferation of anti-immunoglobulin treated normal B cells (Rothstein, 1985; Rothstein, 1986). Also, promyelocytic leukemia HL60 cells cultured in the presence of tumor-promoting phorbol esters undergo rearrangement of the cytoskeleton and differentiate into non-dividing macrophage-like cells (Rovera et al., 1979; Huberman et al., 1979). Although the effects of phorbol esters on cultured cells are many, it is possible that the alteration in the cytoskeletal structure may be involved in the progression of the differentiation pathway (Diamond et al., 1980; Penman et al., 1983).

To define further the role of the cytoskeleton in gene regulation, we have studied the effects of disrupting the cytoskeleton with cytochalasin D on the regulation of a series of genes. We report here that cytochalasin D treatment of exponentially growing HeLa cells rapidly induces the transcription of the c-fos gene. This increase in transcription is accompanied by an increase in c-fos mRNA levels. The effect of cytochalasin D on c-fos gene

expression does not appear to be a general phenomenon since the transcription and steady state levels of several other active and inactive genes are unaffected by the drug treatment. These results imply that the cell monitors the organization and/or composition of the cytoskeleton and adjust the regulation of c-fos gene expression accordingly. This finding is consistent with signal transduction models proposing that the cytoskeleton may be involved in relaying stimuli from the plasma membrane to the nucleus.

Results

Cytochalasin D Increases c-Fos mRNA Levels

Previous reports have suggested that the cytoskeleton plays a role in signal transduction and the regulation of gene expression. To further investigate this functional property of the cytoskeleton, we have disrupted the microfilaments of HeLa S3 cells with cytochalasin D and measured the effects on transcription and mRNA cellular levels of a series of genes. Within minutes of the addition of cytochalasin D, cell morphology is drastically altered as the cytoskeleton rearranges without any apparent quantitative or qualitative loss of protein (Miranda et al., 1974a; Miranda et al., 1974b; Schliwa, 1982; Ornelles et al., 1986). It has been proposed that cytochalasin D acts by binding to actin, causing the capping or fragmentation of the microfilaments (Selden et al., 1980; Lin et al., 1980).

In addition, CD at concentrations equal to or greater than 10 μg/ml releases poly-A^+ RNA from the cytoskeleton in a dose dependent manner (Ornelles et al., 1986). The release of poly-A^+ RNA from the cytoskeleton occurs in a dose dependent manner and results in a stoichiometric inhibition of protein synthesis.

To study immediate gene responses to cytochalasin D, exponentially growing HeLa S3 cells were treated with 10 μg/ml of the drug for only 15 minutes. From each cell culture total cellular RNA for Northern blot analysis and nuclei for in vitro nuclear run-on transcription analysis were isolated as described in Chapter 2. As shown in Figure 7-1, Northern blot analysis reveals a rapid and extensive accumulation of c-fos mRNA in CD treated cells (lane 2). The response of the c-fos gene is in sharp contrast to hCGα mRNA steady state levels which are unaffected by the drug treatment (Fig. 7-1). Quantitation by scanning laser densitometry of several exposures of autoradiograms obtained from four separate experiments indicates an average increase of 20 fold in c-fos mRNA levels (Fig. 7-2). The concentration of cytochalasin D used in these experiments inhibits protein synthesis by 50 percent (Ornelles et al., 1986). It is well established that inhibition of protein synthesis leads to an increase in c-fos mRNA presumably due to increased mRNA stability (Muller et al., 1984; Mitchell et al., 1985; Andrews et al., 1987; Rahmsdorf et al., 1987).

To address the possibility that the stimulation of c-fos mRNA levels was due to the partial inhibition of protein synthesis, we investigated the effect of puromycin at a concentration of 0.4 mM, which inhibits protein synthesis by more than 90 percent (Helms et al., 1984), on c-fos gene expression. Cells cultured in the presence of puromycin for 15 minutes exhibited 9 fold higher levels of c-fos mRNA compared to untreated cell cultures (Fig. 7-1, lane 3; and Figure 8-2). However, cotreatment with CD and puromycin for only 15 minutes resulted in a 49 fold increase in c-fos mRNA levels (Fig. 7-1, lane 4 and Fig. 7-2). The superinduction of c-fos mRNA cellular levels by cytochalasin D and puromycin cotreatment suggests that the stimulation by CD alone is not due solely to its effect on protein synthesis and may be the result of the perturbation of the cytoskeleton.

Kinetics of c-Fos mRNA Accumulation During Cytochalasin D Treatment

Treatment of quiescent BALB/c-3T3 mouse fibroblast cells with growth factors or serum results in a rapid and short lived increase in c-fos mRNA levels (Cochran et al., 1984; Greenberg and Ziff, 1984; Muller et al., 1984). Generally, cellular levels of c-fos mRNA are maximal after 30 minutes of serum/growth factor treatment and return to pre-stimulatory levels within an hour. In contrast to serum

Figure 7-1. Effects of cytochalasin D and puromycin treatment on steady state levels of c-fos and chorionic gonadotropin mRNA.

Exponentially growing HeLa cells were treated with cytochalasin D [10 μg/ml], puromycin [0.4 mM], or cytochalasin D [10 μg/ml] and puromycin [0.4 mM] together for 15 minutes. The cells were harvested and total cellular RNA was isolated. Chorionic gonadotropin (hCGα) and c-fos mRNA levels were determined by Northern blot analysis as described in Chapter 2. Each lane represents 10 μg total cellular mRNA. Lanes: 1) control; 2) cytochalasin D for 15 min.; 3) puromycin for 15 min.; and 4) CD and puromycin for 15 minutes.

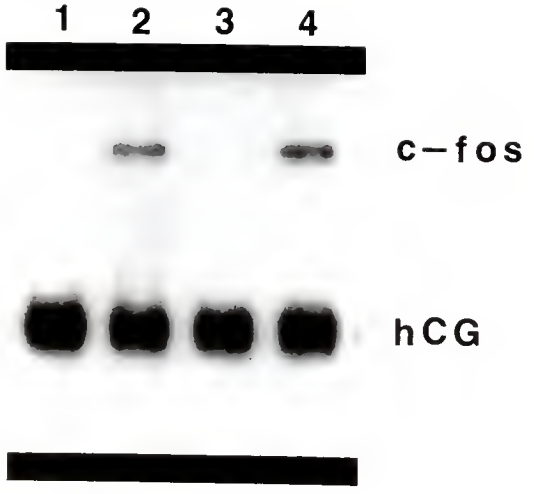
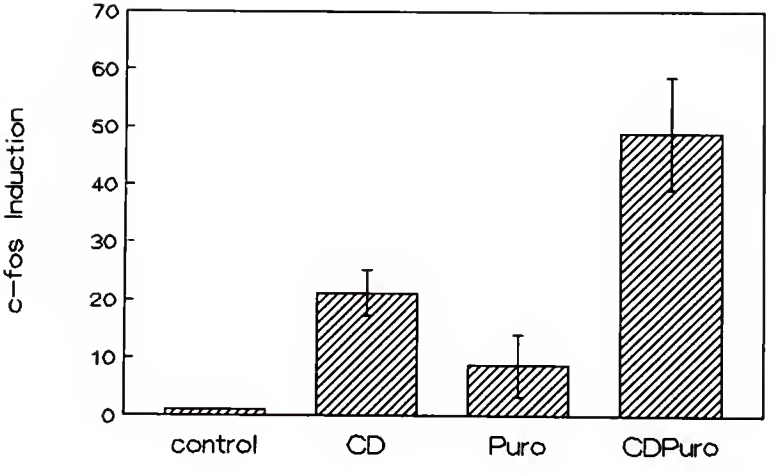

1
2
3
4
c–fos
hCG

Figure 7-2. Densitometric anlaysis of steady state levels of c-fos mRNA during cytochalasin D and puromycin treatments.

Steady state levels of c-fos mRNA during cytochalasin D and puromycin treatments were determined by densitometric analysis of the Northern filters. The values presented represent the average of the minimal fold increase in c-fos mRNA levels during the drug treatments as compared to the control sample. Brackets indicate standard error from three independent experiments.


c-fos Induction
70
60
50
40
30
20
10
0
control
CD
Puro
CDPuro

and growth factor stimulation, inhibition of protein synthesis produces a larger accumulation of c-fos mRNA levels which persists for longer periods of time, usually several hours (Greenberg et al., 1986; Rahmsdorf et al., 1987; Andrews et al., 1987). To determine whether the c-fos gene response to cytochalasin D treatment corresponds to that observed for serum/growth factor stimulation or inhibition of protein synthesis, we examined the kinetics of c-fos accumulation during CD treatment. Northern blot analysis of total cellular RNA isolated from cells treated with 10 μg/ml cytochalasin D for various lengths of time demonstrated that maximal induction of c-fos mRNA, approaching a 40 fold increase over control cultures, occured after 30 minutes and was followed by a sharp decline to near pre-stimulatory levels within one hour (Fig. 7-3, lanes 4-9). In contrast, inhibition of protein synthesis with puromycin resulted in maximal accumulation of c-fos mRNA levels after 90 minutes, nearly 130 fold higher than in untreated cells, and the mRNA levels remained elevated for at least two hours (Fig. 7-3, lanes 16-20). The accumulation of c-fos mRNA in CD and puromycin co-treated cells followed the same time course as observed for puromycin treatment and reached higher levels than with the CD treatment and puromycin treatment combined (Fig. 7-3, lanes 10-15).

Parallel studies were done on serum starved WI38 human diploid fibroblast cells. Stimulation of quiescent cell

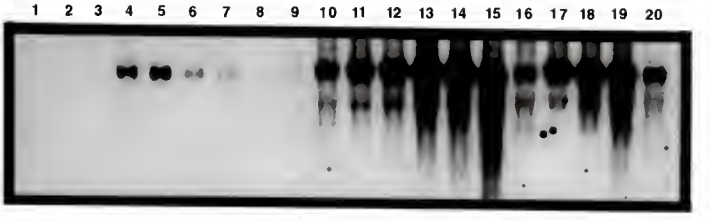
cultures with culture medium containing 10% fetal calf serum resulted in an increase in c-fos mRNA levels for 60 minutes followed by a sharp reduction to backround levels by 120 minutes. Cytochalasin D affected c-fos gene expression with the same kinetics as serum; however, slightly higher levels of c-fos mRNA were measured in CD treated cells. Puromycin treatment or CD and puromycin cotreatment caused a continuous rise in c-fos mRNA levels for two hours, which was the last time point examined. These results indicate that the effects of cytochalasin D on either exponentially growing HeLa cells or quiescent diploid fibroblasts more closely resemble the effects of serum and growth factor stimulation than the inhibition of protein synthesis on the expression of c-fos mRNA levels.

Cytochalasin D Stimulates the Transcription of the c-Fos Gene

To assess the contribution of transcription to the increase in c-fos mRNA levels during CD treatment, we measured the transcriptional activity of the c-fos gene by *in vitro* nuclear run-on transcription analysis as described in Chapter 2. Treatment of HeLa cells with cytochalasin D for 15 minutes resulted in a significant increase in transcription of the c-fos gene. The data presented in Fig. 7-4 represent a twelve fold increase in c-fos transcription in cytochalasin D treated cells compared to untreated,

Figure 7-3. Time course of c-fos mRNA accumulation during cytochalasin D and puromycin treatments in exponentially growing HeLa cells.

HeLa cells were treated with cytochalasin D [10 μg/ml], puromycin [0.4 mM] or cytochalasin D and puromycin for the indicated times. The cells were collected and total cellular RNA was isolated. The levels of c-fos mRNA were determined by Northern blot analysis as described in Chapter 2. Each lane represents 10 μg total cellular RNA. Lanes: 1) control/0 min.; 2) control/60 min.; 3) DMSO control/60 min.; 4) CD/15 min.; 5) CD/30 min.; 6) CD/45 min.; 7) CD/60 min.; 8) CD/90 min.; 9) CD/120 min.; 10) CDPuro/15 min.; 11) CDPuro/30 min.; 12) CDPuro/45 min.; 13) CDPuro/60 min.; 14) CDPuro/90 min.; 15) CDPuro/120 min.; 16) Puro/15 min.; 17) Puro/45 min.; 18) Puro/60 min.; 19) Puro/90 min.; 20) Puro/120 minutes.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
c-FOS

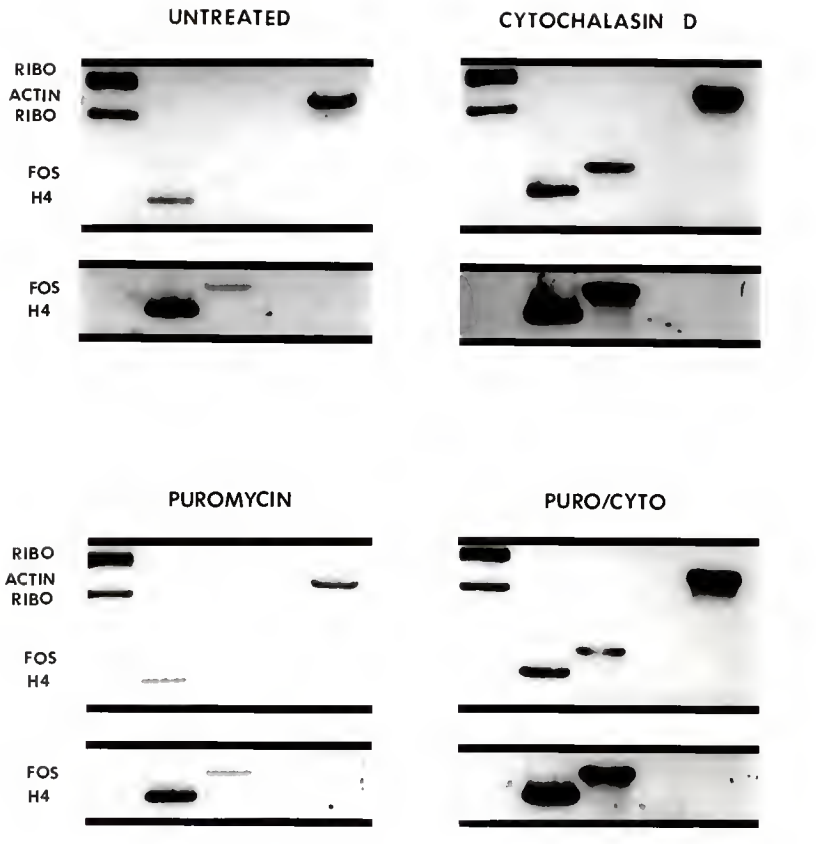
control cells. In contrast, puromycin treatment for 15 minutes did not significantly affect the transcription of the c-fos gene. Cotreatment of HeLa cells with puromycin and cytochalasin D for 15 minutes also stimulated c-fos gene transcription, which indicates that inhibition of protein synthesis during CD treatment does not affect the induction of c-fos transcription.

Tannenbaum and co-workers have demonstrated an increase in actin transcription during CD treatment (1983). In light of this observation, we measured the transcription of the beta-actin gene in our studies as a positive control. Consistent with previous findings, CD increased actin transcription, but only 5 fold over control cells (Fig. 7-4). Cotreatment with CD and puromycin for 15 minutes also increased transcription of the actin gene 5 fold compared to controls and presumably reflects the effects of the cytochalasin D (Fig. 7-4). The cytochalasin D stimulation of c-fos and beta-actin transcription is unaffected by inhibition of protein synthesis.

The elevation in c-fos and actin transcription during cytochalasin D treatment may be part of a general phenomenon in which the transcriptional activity of all genes becomes increased. Alternatively, transcription of ribosomal RNA genes by RNA polymerase I, which represents approximately 50% of the transcriptional activity measured by nuclear run-on assays (Marzluff and Huang, 1985), may be inhibited by

Figure 7-4. Effect of cytochalasin D on transcription of c-fos in HeLa cells.

Exponentially growing HeLa cells were treated with cytochalasin D, puromycin, or CD and puromycin for 15 minutes. Radiolabeled RNAs were prepared by in vitro nuclear run-on transcription and hybridized to Southern blots of electrophoretically separated restriction enzyme digested plasmid DNAs (10^7 cpm/filter). Insert DNAs hybridizing to transcripts from the corresponding genes are indicated (both Ribo signals represent 18S ribosomal RNA transcripts). Untreated, DMSO only; Cytochalasin D, CD [10 μg/ml]; Puromycin, puromycin [0.04 mM]; Puro/Cyto, cotreatment with CD [10 μg/ml] and puromycin [0.04 mM]. Autoradiograms in upper panel for each treatment represent a 28 hour exposure. Autoradiograms in lower panel, indicating Fos and H4 histone transcription, represent a 4 day exposure.


UNTREATED
CYTOCHALASIN D
PUROMYCIN
PURO/CYTO
RIBO
ACTIN
RIBO
FOS
H4
FOS
H4
RIBO
ACTIN
RIBO
FOS
H4
FOS
H4

cytochalasin D and thereby raise the effective concentration of other transcripts. To address these possibilities, we examined the transcription of the 18S rRNA gene as well as other genes during CD treatment. As seen in Fig. 7-4, the transcription of 18S ribosomal RNA and cell cycle dependent histone H4 genes is not significantly affected by cytochalasin D. In addition, CD has no effect on the transcription of genes coding for HLA-B7 (Fig. 7-5), beta-globin and a cell cycle independent H2B histone (data not shown). These results suggest that the increase in transcription of c-fos and beta-actin genes during cytochalasin D treatment is a selective response and not an effect on transcription in general.

Transcriptional Induction of c-Fos During CD Treatment does not Require New Protein Synthesis

The increase in c-fos gene transcription during CD treatment may be a secondary effect of the drug. Cytochalasin D may induce the synthesis of a protein that in turn regulates the transcriptional activity of the c-fos gene. To test this possibility, HeLa cells were pre-treated with puromycin for 15 minutes prior to treatment with cytochalasin D. The cells were then incubated in the presence of both drugs for an additional 15 minutes. Cell cultures treated with puromycin alone were incubated for 30 minutes in the presence of the inhibitor while control cells

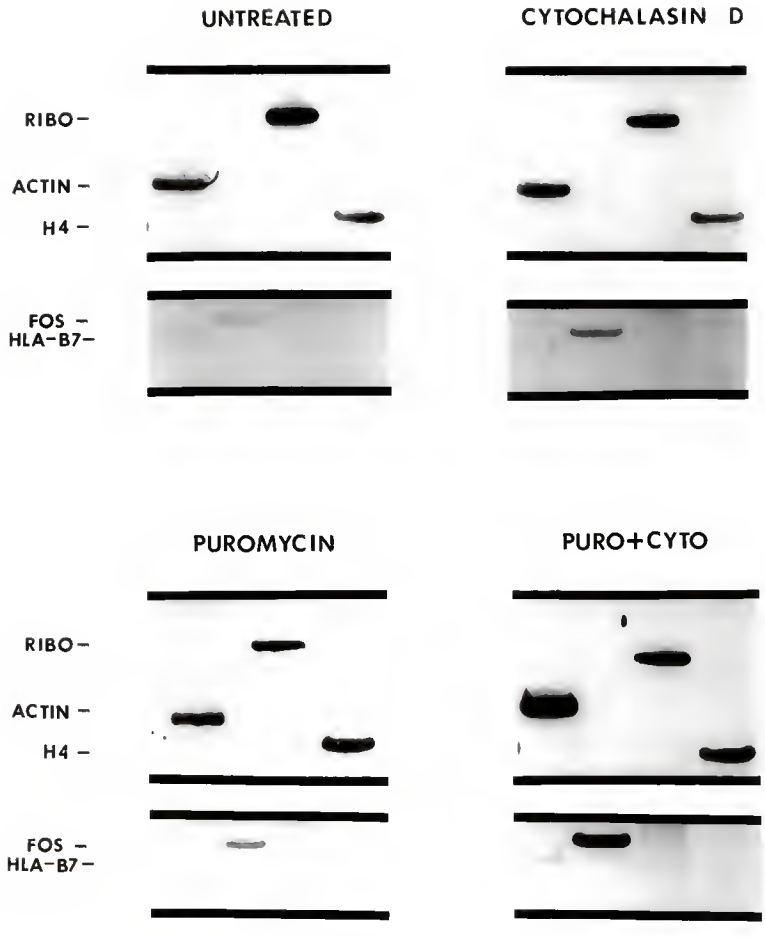
were incubated in an equivalent concentration of DMSO for 30 minutes. Treatment of HeLa cells with cytochalasin D for 15 minutes resulted in the expected stimulation of c-fos transcription (Fig. 7-5). Pretreatment of HeLa cells with puromycin did not block the CD induced stimulation of c-fos transcription and in fact, markedly increased the transcription of the c-fos gene (Fig. 7-5). These results suggest that the transcription factors are present within the cells prior to cytochalasin D treatment, which is consistent with previous findings that the factors involved in the increase in c-fos transcription during serum stimulation of quiescent fibroblasts are pre-existent (Sassone-Corsi and Verma, 1987; Greenberg et al., 1986).

Cytochalasin D Releases c-Fos mRNA from the Cytoskeleton into the Soluble Phase

The marked increase in c-fos mRNA cellular levels during cytochalasin D treatment is not completely accounted for by the increase in c-fos transcription and may be partly a result of enhanced mRNA stability. Previous results indicated that cytochalasin D [>10 μg/ml] releases poly-A^+ RNA from the cytoskeleton into the soluble phase in a dose dependent manner (Ornelles et al., 1986). The stabilization of c-fos mRNA during cytochalasin D treatment may therefore be a consequence of an alteration in mRNA subcellular location. To begin addressing this possibility, we have

Figure 7-5. Effect of protein synthesis inhibition on cytochalasin D induction of c-fos transcription in HeLa cells.

Exponentially growing HeLa cells were treated with cytochalasin D, puromycin, or puromycin and CD for the indicated times. Radiolabeled RNAs were prepared by in vitro nuclear run-on transcription and hybridized to Southern blots of electrophoretically separated restriction enzyme digested plasmid DNAs (10^7 cpm/filter). Insert DNAs hybridizing to transcripts from the corresponding genes are indicated. Untreated, DMSO for 15 min.; Cytochalasin D, CD [10 μg/ml] for 15 min.; Puromycin, puromycin [0.04 mM] for 30 min.; Puro + Cyto, puromycin [0.04 mM] for 30 min. and CD [10 μg/ml] for the last 15 minutes.


UNTREATED
CYTOCHALASIN D
RIBO –
ACTIN –
H4 –
FOS –
HLA-B7–
PUROMYCIN
PURO+CYTO
RIBO –
ACTIN –
H4 –
FOS –
HLA-B7–

examined the subcellular location of c-fos mRNA, with respect to its association with the cytoskeletal structure, in both control and cytochalasin D treated cells.

Northern blot analysis of cytoskeletal and soluble phase RNAs from untreated HeLa cells demonstrated that approximately 80% of c-fos mRNA is associated with the cytoskeletal structure (Figure 5-2 and Table 5-2). Cytochalasin D treatment efficiently disrupted this association and released c-fos mRNA from the cytoskeleton into the soluble phase. Greater than 70% of the c-fos mRNA accumulated in the soluble phase in cytochalasin D treated cells. Cytochalasin D and puromycin cotreatment also preferentially increased c-fos mRNA levels in the soluble phase. Nearly 80% of the c-fos mRNA was localized in the soluble fraction isolated from CD and puromycin co-treated cells. Consistent with the work of Ornelles et al. (1986), puromycin treatment alone, which also increased c-fos mRNA levels, had essentially no effect on the cytoskeletal and soluble phase distribution of the mRNA. Approximately 85% of the c-fos mRNA remained associated with the cytoskeleton in puromycin treated cells. These results indicate that the majority of the c-fos mRNA synthesized during cytochalasin D treatment is localized in an altered subcellular region. Accumulation of c-fos mRNA in an unnatural subcellular compartment may physically remove it from the regulatory factors that are involved in its stability.

Discussion

Previous results suggest that the organization of the cytoskeleton may influence gene expression and ultimately cell growth and differentiation. We present evidence here supporting the role of the cytoskeleton in regulating gene expression. Specifically, disruption of the microfilaments with cytochalasin D results in a rapid and dramatic accumulation of c-fos mRNA. The induction of c-fos gene expression during cytochalasin D treatment is accompanied by an increase in transcription of the c-fos gene as directly determined by *in vitro* nuclear run on analysis.

The effect of cytochalasin D treatment on transcription is not limited to the c-fos gene. Consistent with previous results actin transcription was also elevated in cytochalasin D treated cells (Tannenbaum and Godman, 1983). However, the stimulation of transcription of the c-fos and actin genes was not a consequence of increased transcription in general. RNA polymerase II transcription of several genes, such as histone H4 (cell cycle dependent), histone H2B (cell cycle independent), and HLA-B7 histocompatability antigen, was unaffected in cells treated with cytochalasin D. In addition, cytochalasin D had no effect on transcription of the 18S ribosomal RNA genes by RNA polymerase I or transcription of the beta-globin gene, a transcriptionally inactive gene in HeLa cells.

Regulation of c-fos gene expression is controlled at both the transcriptional and posttranscriptional levels. A variety of agents such as serum, growth factors and differentiation factors transcriptionally induce c-fos gene expression in a rapid and transient manner (Greenberg and Ziff, 1984). In contrast, inhibition of protein synthesis with cycloheximide or heat shock results in a slower but more persistent accumulation of c-fos mRNA with minimal or no effect on c-fos transcription (Andrews et al., 1987; Rahmsdorf et al., 1987). The increase in c-fos mRNA levels during inhibition of protein synthesis appears to be largely posttranscriptionally regulated through the stabilization of the c-fos mRNA. Previous results have demonstrated that cytochalasin D can inhibit protein synthesis in a dose dependent manner by releasing poly-A^+ RNA from the cytoskeleton into the soluble phase as mRNP particles (Ornelles et al., 1986). The concentration of cytochalasin D used in this study was sufficient to inhibit protein synthesis by 50 percent. However, our results suggest that the induction of c-fos gene expression by cytochalasin D treatment is not a result of the partial inhibition of protein synthesis. Cytochalasin D and puromycin cotreatment resulted in roughly an additive increase in c-fos gene transcription, suggesting the effects of the two drugs on transcription are due to different mechanisms. Consistent with this reasoning, the cellular level of c-fos mRNA in

cytochalasin D and puromycin co-treated cells is greater than the levels of c-fos mRNA in CD treated and puromycin treated cells combined. Furthermore, the time course of c-fos mRNA accumulation in cytochalasin D treated cells is more rapid and shorter lived than that observed in puromycin treated cells.

Stimulation of c-fos transcription by cytochalasin D treatment can occur during inhibition of protein synthesis and therefore appears to be dependent on factors that are already present within the treated cells. Pretreatment with puromycin for 15 minutes followed by the addition of cytochalasin D for 15 minutes results in a marked increase in c-fos transcription (Fig. 7-5). Furthermore, inhibition of protein synthesis for 30 minutes with puromycin alone results in a significant increase in c-fos transcription. These results are consistent with previous reports suggesting that the c-fos gene is negatively regulated by a labile factor (Mitchell et al., 1985; Greenberg et al., 1986; Sassone-Corsi and Verma, 1987).

The increase in the cellular levels of c-fos mRNA during cytochalasin D treatment is greater than the measured increase in the transcription of the gene and may be due to the stabilization of c-fos mRNA under these conditions. However, it is possible that during cytochalasin D treatment the mechanism involved in the turnover of c-fos mRNA remains operative despite the reduced levels of protein synthesis.

Previous studies on cytochalasin D treated HeLa cells demonstrated the selective destabilization of histone mRNA during inhibition of DNA synthesis (unpublished data). This event is strictly dependent on protein synthesis and demonstrates the competence of cytochalasin D treated cells to carry out certain posttranscriptional processes. The apparent stabilization of c-fos mRNA during cytochalasin D treatment may therefore be a result of the release of the mRNA from the cytoskeleton in monosome or mRNP form. Release of c-fos mRNA from the cytoskeleton into the soluble phase could physically separate the message from the factors involved in its turnover. These regulatory factors may be sequestered and concentrated by the cytoskeleton or an integral component of the ribosome.

The mechanism of induction of c-fos gene expression by cytochalasin D treatment is not known. Many of the inducers of c-fos transcription studied to date appear to effect protein kinase C and/or adenylate cyclase enzyme activities (Verma and Sassone-Corsi, 1987). The induction of c-fos gene expression by cytochalasin D may also occur as an indirect effect on protein kinase C or adenylate cyclase levels although this has not yet been determined. Alternatively, the disruption of the cytoskeleton by cytochalasin D may also perturb the nuclear matrix which may in turn affect transcription. However, a model which includes the alteration in transcription as a result of the perturbation

of the nuclear matrix must explain the selective activation of c-fos and actin genes and therefore does not appear likely. In contrast, stimulation of c-fos gene expression by cytochalasin D may be a result of the alterations in the cytoskeleton due to the disruption of the microfilaments. Analogous to the work presented here, it has recently been reported that the disruption of the microtubules with colchicine, nocodazole, or vinblastine induces the transcription of chloramphenicol acetyl transferase genes containing promoter elements from the human c-fos or beta-actin gene (Ng, 1989). Interestingly, sequence analysis indicates that actin and c-fos genes contain similar transcription consensus sequences, referred to as serum response elements, located in their promoter regions (Mohun and Garret, 1987). The coordinate induction of c-fos and actin transcription during cytochalasin D or colchicine treatment may be mediated by the interaction of the same or similar trans acting factor(s) with the serum response element.

Several reports have postulated that the cytoskeleton is involved in signal transduction (Rothstein, 1985; Rebillard et al., 1987; Blum and Wicha, 1988). For example, Rebillard and co-workers (1987) reported that the stimulation of quiescent Swiss 3T3 cells with epidermal growth factor (EGF) and insulin results in the rapid accumulation of c-fos mRNA. The increase in c-fos gene

expression during EGF and insulin cotreatment can be inhibited by disruption of the cytoskeleton with cytochalasin D. This result is consistent with the hypothesis that the cytoskeleton may be involved in relaying the EGF/insulin signal from the cell surface to the nucleus. The results presented here describing the stimulation of c-fos gene expression with cytochalasin D, although contrary to Rebillard's results, are consistent with their interpretation that the nucleus monitors the integrity of the cytoskeleton and adjusts the expression of particular genes accordingly. Rather than cytochalasin D inhibiting a signal from the cell surface to the nucleus, the drug in our hands may mimic the signal to stimulate c-fos gene expression. Cell type, culture conditions and drug dosage effects may explain the differences observed between our results and those of Rebillard's et al. (1987).

In summary, cytochalasin D stimulates c-fos gene expression through increased transcription and mRNA stability. The effect of cytochalasin D on c-fos gene expression may be in response to the alterations in the cytoskeleton and cell shape. Whether other elements of the cytoskeleton such as the intermediate filaments can elicit the same response remains to be determined. Taken together with previous reports, the ability of cytochalasin D to stimulate c-fos in quiescent fibroblasts with the same kinetics and virtually to the same extent as serum supports the role of the cytoskeleton in signal transduction.

CHAPTER 8

SUMMARY AND FUTURE CONSIDERATIONS

The initial emphasis of the work presented in this dissertation was focused on studying the posttranscriptional regulation of histone gene expression. When this project was undertaken little was known concerning the coupling of histone mRNA stability to DNA replication. It was clear that the destabilization of histone mRNA during inhibition of DNA synthesis was selective and required ongoing protein synthesis (Butler and Mueller, 1973; Gallwitz, 1975; Stahl and Gallwitz, 1977; Stimac et al., 1983; Baumbach et al., 1984; Helms et al., 1984). In addition, it was known that the kinetics of histone gene expression were extremely rapid which is evident by the appearance of translatable histone mRNAs on polysomes within minutes following transcription (Borun et al., 1967) and the equally rapid transfer of newly synthesized histone protein into the nucleus (Spaulding et al., 1966; Robbins and Borun, 1967). At that time, several research groups were providing evidence that suggested the cell is organized in a highly ordered manner. Penman and co-workers, while studying the structural and functional properties of the cytoskeleton, were developing the

perspective that cytostructure can form subcellular compartments that are independent of membrane envelopes (Fulton et al., 1980; Cervera et al., 1981). Furthermore, Singer and co-workers, using in situ hybridization techniques, were demonstrating the localization of specific mRNAs in discrete areas of the cytoplasm (Lawrence and Singer, 1986).

Based on these observations and the information available concerning histone gene expression, we proposed that histone mRNAs and the factors that are involved in their selective destabilization during inhibition of DNA synthesis are co-compartmentalized in a perinuclear region. This model predicted that the subcellular location of histone mRNA plays a role in the coupling of histone mRNA stability with DNA replication.

To address this question, we first determined the normal subcellular location of histone mRNA. Subcellular fractionation and Northern blot analysis demonstrated that histone mRNAs are naturally located on nonmembrane-bound polysomes that are associated with the cytoskeleton (Chapter 3). The subcellular localization of histone mRNA was then altered by incorporating nucleotide sequences coding for a signal peptide into an H3 histone mRNA (Chapter 4). The modified histone gene, when transfected into HeLa cells, expressed a signal peptide-histone fusion mRNA which was directed to membrane-bound polysomes. The histone fusion

mRNA, in contrast to endogenous histone mRNA, was not efficiently destabilized when DNA synthesis was interrupted with hydroxyurea. Site directed mutagenesis analysis of the histone fusion gene and subsequent *in vivo* studies indicated that the uncoupling of the histone fusion mRNA stability from DNA replication was due to the altered subcellular location and was not due to the perturbation of mRNA structure.

It has since been demonstrated by *in situ* hybridization analysis that histone mRNAs are localized in "grape like clusters" throughout the cytoplasm (Lawrence *et al.*, 1988). In this regard, the initial proposal that histone mRNAs are compartmentalized in a region close to the nucleus was incorrect. The non-uniform distribution of histone mRNAs within the cytoplasm, however, suggests that histone mRNAs are compartmentalized. The stability of histone fusion mRNA during inhibition of DNA synthesis, due to the association of the mRNA with membrane-bound polysomes, is consistent with the possibility that these compartments are involved in the posttranscriptional regulation of histone gene expression.

The proposal that histone protein synthesis is autogenously regulated provides one possible explanation for the role of the subcellular compartments in the posttranscriptional regulation of histone gene expression. As proposed by Butler and Mueller (1973) and several other

research groups (Stein and Stein, 1984; Wu and Bonner, 1985), a critical concentration of unbound histone protein may be required for the destabilization of histone mRNA during inhibition of DNA synthesis. Localization of histone mRNA in clusters may create pockets within the cytoplasm in which the unbound histone protein could reach the necessary concentration for the destabilization of histone mRNA to occur.

The alteration in subcellular location of the histone fusion mRNA has been demonstrated by subcellular fractionation and S1 nuclease protection analysis (Chapter 4). It remains to be determined by in situ hybridization analysis whether the spatial distribution of the histone fusion mRNA has been significantly altered compared with that of endogenous histone mRNA. Furthermore, identification and purification of the components involved in the posttranscriptional regulation of histone gene expression may provide the opportunity to monitor directly the subcellular localization of these factors by immunofluorescence, cell fractionation and biochemical analysis.

Another aspect concerning the localization of histone mRNA in the cytoplasm, which warrants additional consideration, is whether a single class or multiple classes of histone mRNAs are contained within a cluster. For example, a particular cluster may contain only H3 histone

mRNA, H3 and H4 histone mRNA together, or any combination of groupings of the different classes of histone mRNAs. The pattern for these groupings may be important in the transport of the newly synthesized histone proteins into the nucleus and/or the coordinate regulation of histone protein synthesis.

Although unrelated to the problems addressed here, it would be interesting from the standpoint of studying the process of protein secretion to determine the subcellular localization of the histone fusion protein. Examination of subcellular fractions isolated from HeLa cells which are expressing the signal peptide-histone fusion gene, by standard biochemical techniques, such as acid extraction and triton-urea gel electrophoresis, would provide insight into how the fusion protein is processed and where the fusion protein is located.

In studying the subcellular location of histone mRNA, it was determined that histone mRNA is predominantly associated with the cytoskeleton and that this association is dependent on the integrity of the microfilaments (Chapters 3 and 5). Cytochalasin D treatment, which disrupts the actin filaments, releases histone mRNA from the cytoskeleton into the soluble phase. This result is consistent with the observation of Penman and co-workers that heterogeneous poly-A^+ RNA is released from the cytoskeleton in cytochalasin D treated HeLa cells (Ornelles *et al.*, 1986).

The release of histone mRNA from the cytoskeleton by cytochalasin D treatment presented the opportunity to study the role of the cytoskeleton in the posttranscriptional regulation of histone gene expression. Histone mRNA was released into the soluble phase with moderate amounts of cytochálasin D [10 ug/ml], since higher levels of CD significantly inhibit protein synthesis. The cells were then treated with hydroxyurea to inhibit DNA replication. These treatments resulted in the destabilization of histone mRNA in the soluble fraction to the same extent and with the same kinetics as the destabilization of histone mRNA in the cytoskeleton fraction. One interpretation of this result is that the cytoskeleton is not required for histone mRNA destabilization during inhibition of DNA synthesis. In addition, Ornelles et al. (1986) reported that CD treatment of HeLa cells releases poly-A^+ RNA from the cytoskeleton into the soluble phase in monosome or mRNP form, which suggests that histone mRNA in the soluble phase does not need to be translated to be destabilized during inhibition of DNA synthesis. This result is in contrast to the genetic studies that indicate histone mRNA must be translated to within close proximity of the translation stop codon for destabilization to occur (Graves et al., 1987; Capasso et al., 1987). The release of histone mRNA into the soluble phase as a monosome or mRNP during cytochalasin D treatment has not yet been determined and additional studies will be

necessary to clarify these interpretations of the cytochalasin D-hydroxyurea experiments. For example, sucrose density gradient analysis of soluble phase RNA from cytochalasin D treated cells should demonstrate whether histone mRNA is released from the cytoskeleton in the same manner as poly-A^+ RNA. Furthermore, gel filtration studies as described by Adams et al. (1983) on the composition of the histone mRNA in the soluble phase after cytochalasin D treatment should also provide information on whether the histone mRNA, although no longer anchored to the cytoskeleton, may still be complexed with cytoskeletal components. These cytoskeletal components may be necessary for histone mRNA destabilization during inhibition of DNA replication.

The distribution of HLA-B7 mRNA between the cytoskeleton and soluble fractions during cytochalasin D and hydroxyurea cotreatment was also analyzed as an internal control for equal loading of the RNA. Surprisingly, in view of the results of Ornelles et al. (1986), cytochalasin D treatment failed to release HLA-B7 mRNA from the cytoskeleton (Chapter 5). Subsequent studies demonstrated that polysomal HLA-B7 mRNA, as well as other membrane-bound polysomes, are released from the cytoskeleton during cytochalasin D and puromycin cotreatment (Chapter 5). Puromycin alone or cytochalasin D and cycloheximide cotreatment does not release membrane-bound polysomes from

the cytoskeleton. These results suggest that one mechanism for the attachment of membrane-bound polysomes to the cytoskeleton is through the interaction of the polysome complex with the endoplasmic reticulum. This interaction appears to involve the nascent polypeptide; however other possibilities may exist.

The relocation of signal peptide-histone fusion mRNA to membrane-bound polysomes presented the opportunity to study the cytoskeletal interactions of membrane-bound polysomal mRNAs in more detail. Our model predicted that histone fusion mRNA would remain attached to the cytoskeleton during CD treatment and would be released into the soluble phase during CD and puromycin cotreatment. As seen in Chapter 6, the signal peptide-histone fusion mRNA remained attached to the cytoskeleton during cytochalasin D treatment as well as during cytochalasin D and puromycin cotreatment. The retention of signal peptide-histone fusion mRNA on the cytoskeleton under these conditions demonstrates that the histone fusion mRNA is associated with the cytoskeleton in a manner that is different from that observed for wild type histone mRNA, HLA-B7 membrane-bound polysomal RNA and heterogeneous poly-A^+ RNA. Although it is well documented that mRNAs are associated with the cytoskeleton, there are no known cytoskeleton attachment sites identified to date. Dissection of the signal peptide-histone fusion mRNA, by deletion and site directed mutation analysis, should prove

useful in the identification of an mRNA-cytoskeleton attachment site.

The role of the cytoskeleton structure in influencing gene expression is supported by the observation that cytochalasin D treatment resulted in a rapid and dramatic increase in c-fos gene transcription with a concomitant increase in c-fos mRNA cellular levels (Chapter 7). Consistent with previous results (Tannenbaum and Godman, 1983), CD treatment also increased beta-actin gene transcription. However, these modifications in gene expression appear to be selective and not a general phenomenon, since the transcription of several other genes was unaffected by the drug treatment. The increase in c-fos mRNA cellular levels was larger than the increase in c-fos gene transcription and may be due to a stabilization of the c-fos message under these conditions. Cell fractionation and Northern blot analysis demonstrated that c-fos mRNA is associated with the cytoskeleton in control cells and accumulates in the soluble phase in cytochalasin D treated cells (Chapter 5). Stabilization of c-fos mRNA may be due to the altered subcellular location of the message during cytochalasin D treatment. Additional studies are needed to define the molecular basis for the role of the cytoskeletal structure in the transcriptional and posttranscriptional regulation of c-fos gene expression.

In summary, the work presented in this dissertation is consistent with the hypothesis that subcellular compartments and cell structure play an important role in the regulation of gene expression. The influence of mRNA subcellular location on the posttranscriptional control of histone gene expression and possibly c-fos gene expression, indicates that regulatory factors may be concentrated and sequestered in specific regions or by specific structures. The differential association of mRNA with the cytoskeleton may be a mechanism for localization of mRNA in a particular region of the cell, which in turn may affect the posttranscriptional regulatory process. Lastly, the effect of disrupting the microfilaments with cytochalasin D on c-fos gene and beta-actin gene expression suggests that the nucleus can transcriptionally respond in a selective manner to the structural organization of the cytoskeleton.

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BIOGRAPHICAL SKETCH

Gerard Paul Zambetti was born in Queens, New York, on August 4, 1958. He attended Archbishop Molloy High School (Queens, New York) and graduated in the spring of 1976. He then attended the State University of New York at Plattsburgh and graduated with a Bachelor of Science degree in biochemistry and biophysics in the spring of 1980. He then studied bacteriophage Lambda DNA replication and gene regulation with Dr. Robert Shuster at Emory University (Atlanta, Georgia) and graduated with a Master of Science degree in biochemistry. He continued to research the regulation of gene expression with Drs. Janet and Gary Stein in the Department of Immunology and Medical Microbiology at the University of Florida. In the fall of 1984, he married Stacey Ann Chapman in Atlanta, Georgia, and on May 6, 1987, they were blessed with a beautiful daughter, Shannon Kelly Zambetti.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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